Nuevas estrategias farmacológicas en el tratamiento de la psoriasis

New pharmacological strategies for the treatment of psoriasis

Tesis Doctoral
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VNIVERSITAT DE VALÈNCIA
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presentada por:

Rosa María Andrés Ejarque

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VNIVERSITAT DE VALÈNCIA

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CERTIFICAN:

Que el trabajo presentado por la Licenciada **Rosa María Andrés Ejarque**, titulado ***"Nuevas estrategias farmacológicas en el tratamiento de la psoriasis"***, ha sido realizado en el Departament de Farmacologia de la Universitat de València, bajo nuestra dirección y asesoramiento.

Concluido el trabajo experimental y bibliográfico, autorizamos la presentación de esta Tesis Doctoral para que sea juzgada por el Tribunal correspondiente.

Valencia, a 15 de Abril de 2013.

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Abreviaturas

AMPc	Adenosin monofosfato cíclico
BTH	4-benzo[*b*]thiophen-2-yl-3-bromo-5-hydroxy-5*H*-furan-2-one 4-benzo[*b*]tiofen-2-il-3-bromo-5-hidroxi-5*H*-furan-2-ona
CCL	Chemokine (C-C motif) ligand: quimiocina (C-C) ligando
CI_{50}	Concentración inhibidora 50
CK	Citoqueratina
CS	Chondroitin sulfate, condroitín sulfato
CXCL-1	Chemokine (C-X-C motif) ligand 1: quimiocina (C-X-C) ligando 1
DC	Dendritic cell, célula dendrítica
EMSA	Electrophoretic mobility shift assay: ensayo de movilidad electroforética retarda
ERK	Extracellular signal-regulated kinases: cinasas reguladas por señales extracelulares
GWAS	Genome-wide association study: estudio de asociación del genoma completo
IFN	Interferon, interferón
IL	Interleukin, interleucina
IMQ	Imiquimod
Jak	Janus kinase: Janus cinasa
JNK	c-Jun N-terminal cinasas
LTB_4	Leucotrieno B_4
MAPK	Mitogen-activated protein kinase: proteín cinasas activadas por mitógenos
MHC-1	Major histocompatibility complex 1: complejo mayor de histocompatibilidad 1
mPGEs-1	Prostaglandina E sintasa microsomal 1
NECA	5-N-etil-carboxamidoadenosina
NF-*κ*B	Nuclear factor *κ*B: factor de transcripción nuclear *κ*B

NHK	Normal human keratinocytes: queratinocitos humanos normales
NO	Nitric oxide: óxido nítrico
PASI	Psoriasis Area Severity Index: índice de intensidad y gravedad de la psoriasis
pDC	Plasmacytoid dendritic: células dendríticas plasmocitoides
PGE_2	Prostaglandin E_2, prostaglandina E_2
PIAS	Protein inhibitor of activated STAT: proteína inhibidora de STAT activado
PKC	Proteína cinasa C
PLA_2	Fosfolipasa A_2
PSORS	Regiones de susceptibilidad genética para la psoriasis
Ser727	Residuo de serina presente en la posición 727 de STAT3
SNP	Single-nucleotide polymorphism: polimorfismos de nucleótido simple
SOCS	Suppressor of cytokine signalling: proteína supresora de la señalización por citocinas
STAT	Signal transducer and activator of transcription: transductor de señal y activador de la transcripción
TLR	Toll-like receptor: receptor tipo Toll
TNFα	Tumor necrosis factor α: Factor de necrosis tumoral α
TNFR	Receptor de TNFα
TPA	12-*O*-tetradecanoylphorbol-13-acetate, 12-*O*-tetradecanoilforbol-13-acetato
Tyk2	Tyrosine kinase 2: tirosina cinasa 2
Tyr705	Tyrosine residue in the position 705 of STAT3 Residuo de tirosina presente en la posición 705 de STAT3
VEGF	Factor de crecimiento endotelial vascular

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Resumen

La psoriasis es una enfermedad inflamatoria crónica caracterizada por la inflamación de la piel, que viene acompañada por hiperproliferación de los queratinocitos, infiltración leucocitaria dérmica y una angiogénesis incrementada. Los mecanismos patológicos que dirigen esta patología incluyen tanto respuestas de la inmunidad innata como de la adaptativa. En la presente tesis doctoral se ha ahondado en el estudio de la respuesta fisiopatológica de los queratinocitos a los cambios que se producen en el entorno de una placa psoriásica, con el objetivo de aportar evidencia de relevancia para el desarrollo de nuevos tratamientos farmacológicos. De este modo, se ha puesto de manifiesto que los mediadores proinflamatorios presentes en el contexto psoriásico son capaces de alterar la expresión de los receptores de adenosina, nucleósido implicado en el efecto antiinflamatorio del metotrexato, produciéndose una disminución de los receptores A_{2B} y un aumento de los A_{2A}. Así mismo, se ha demostrado que en piel psoriásica lesional existe una sobreactivación de los transductores de señales y activadores de la transcripción (STAT) 1 y 3, pese a que se produce una disminución mayoritaria de la expresión de las Janus cinasas (Jak). Además, se ha caracterizado el papel de diversos mediadores proinflamatorios en la modulación de estas vías de señalización.

Recientemente se ha puesto de manifiesto la importancia de STAT3, así como del factor de transcripción nuclear κB (NF-κB) en la patogénesis de la psoriasis. Por ello, y con el fin de obtener nuevos fármacos útiles para el tratamiento de la enfermedad, en la presente tesis hemos evaluado el potencial efecto antipsoriásico de diversos derivados de origen natural en base a su capacidad para inhibir la activación de estos dos factores de transcripción.

Para ello, iniciamos nuestro estudio mediante un screening previo en la línea de macrófagos murinos RAW 264.7 de una serie de moléculas obtenidas en su mayoría de esponjas marinas, así como del condroitín sulfato (CS), proteoglicano de amplio uso en la terapéutica de la osteoartritis. La siguiente fase de nuestro proceso de

cribado se realizó en cultivos primarios de queratinocitos humanos, en los que se evaluó la capacidad para inhibir la liberación de las citocinas factor de necrosis tumoral α (TNFα) e interleucina (IL)8. Estos estudios preliminares nos llevaron a la selección del derivado semisintético 4-Benzo[*b*]tiofen-2-il-3-bromo-5-hidroxi-5*H*-furan-2-ona (BTH) y del CS para su estudio en profundidad.

En la última fase de nuestro estudio, el pretratamiento de queratinocitos primarios humanos con el CS inhibió la activación del NF-κB y, en consecuencia, la liberación de citocinas proinflamatorias. Además, disminuyó la translocación al núcleo de STAT3 así como su actividad transcripcional, demostrando su potencial interés como nuevo fármaco antipsoriásico.

Por otro lado, el derivado semisintético BTH también disminuyó la liberación de citocinas y quimiocinas proinflamatorias gracias a su efecto inhibidor de la activación del NF-κB. Además, disminuyó la activación de STAT3 a través del bloqueo de la fosforilación en el residuo de tirosina 705 (Tyr705). Estos resultados fueron confirmados *in vivo* mediante dos modelos de psoriasis en ratón: la hiperplasia epidérmica inducida por 12-*O*-tetradecanoilforbol-13-acetato (TPA) y la inflamación psoriásica inducida por imiquimod. En ambos modelos, la administración tópica del BTH previno el desarrollo de lesiones psoriásicas a través de la supresión de la fosforilación del NF-κB y el STAT3, confirmando el potencial de esta molécula como nuevo fármaco antipsoriásico.

Globalmente, la presente tesis arroja nueva luz sobre el papel de la adenosina y las vías de señalización del NF-κB y el STAT3 en la patogénesis de la psoriasis y pone de manifiesto la eficacia de moléculas sencillas de origen natural en la paliación de su curso, sugiriendo el potencial interés de estas estrategias farmacológicas en el desarrollo de nuevos abordajes terapéuticos para el tratamiento de esta enfermedad.

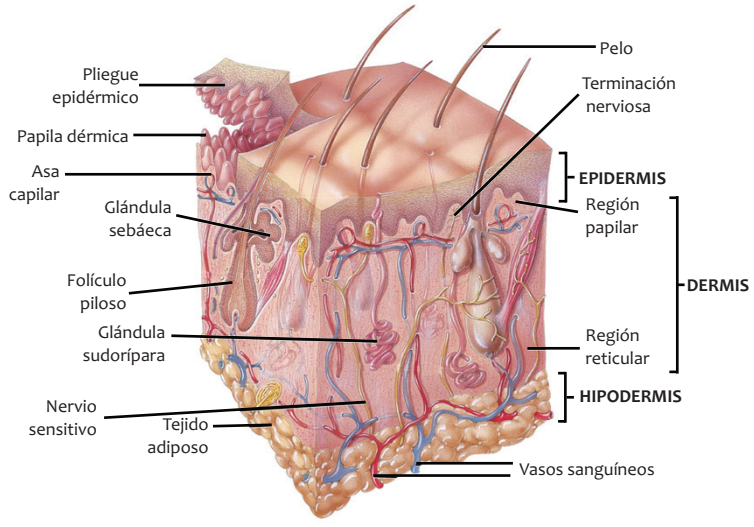
Introducción

1. La Piel

Con un espesor medio de 2 mm, la piel es la principal barrera protectora del organismo, que salvaguarda el ambiente interno, cálido y húmedo, frente el esencialmente hostil, frío y seco ambiente externo en el que vivimos. La piel es el órgano más visible del organismo y el más importante tanto en superficie, llegando a abarcar un área de 2 m^2, como en peso, aportando el 16% de la masa corporal.

Aparte de protegernos, la piel juega un papel fundamental en el control de la temperatura corporal y de la presión arterial, posee funciones endocrinas y exocrinas y contiene numerosos receptores sensoriales que nos ayudan a comunicarnos con el entorno. Por ello, una piel sana es imprescindible para el buen funcionamiento del organismo.

Desde el punto de vista estructural, la piel está dividida en dos capas principales: la dermis y la epidermis. Existe una tercera capa, llamada hipodermis, que sirve como soporte y conexión con el resto del organismo (Figura 1).



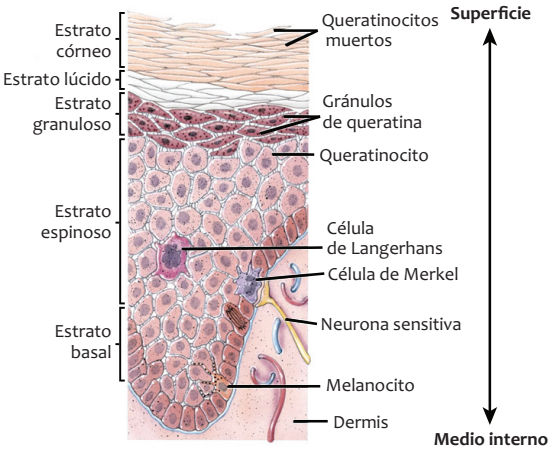
Adaptado de Tortora y Derrickson (2006)

Figura 1. Corte transversal de piel y tejido subcutáneo.

1.1 Epidermis

Las capas más externas de la piel conforman la epidermis, tejido metabólicamente activo, que sintetiza lípidos, glicosaminoglicanos y ceramidas y contiene todos los componentes necesarios para formar la barrera protectora. Constituida en un 95% por queratinocitos, la epidermis alberga también otros tipos celulares, como son las células de Langerhans, los melanocitos y las células de Merkel, imprescindibles para el ejercicio de las funciones accesorias de la piel. Las células de Langerhans, formadas en la médula ósea, suponen la primera barrera frente a agresores inmunológicos. La epidermis no está irrigada, las células en las capas más internas se nutren por difusión de los capilares que llegan hasta las capas más próximas de la dermis (Figura 1).

Varias capas de queratinocitos, en distintos estadios de desarrollo, forman la epidermis. Las más externas están aplanadas, por lo que se considera un epitelio escamoso estratificado. La capa más interna, la basal, está formada por células madre epidérmicas que se dividen por mitosis, dando lugar a dos células hijas: una que permanecerá en la capa basal y otra que avanzará a través de las capas epidérmicas hasta la superficie. En condiciones normales, esta progresión dura alrededor de 28 días y va acompañada de cambios fundamentales en el contenido celular, que dan lugar a las diferentes capas visibles al microscopio (Figura 2).



Adaptado de Tortora y Derrickson (2006)

Figura 2. La epidermis consiste en un epitelio escamoso estratificado.

A medida que ascienden hacia el exterior, los queratinocitos comienzan a rellenarse de una sustancia granular llamada queratina, pierden agua y se aplanan. La producción de queratina comienza en el estrato espinoso y continúa en el estrato granuloso, donde las células pierden el núcleo e inician un proceso de apoptosis. El estrato lúcido y el estrato córneo están compuestos de células muertas, estrechamente empaquetadas y rodeadas de lípidos. Estas capas protegen las más internas y son finalmente eliminadas por descamación conforme nuevos queratinocitos las van reemplazando.

Debido a que el ciclo celular normal de una queratinocito (de mitosis a mitosis) dura aproximadamente 6 días, en condiciones normales no todas las células de la capa basal están activas a la vez. Sin embargo, en determinadas condiciones patológicas, como la psoriasis, el tiempo de tránsito de los queratinocitos se acorta de 28 a 7 días y su maduración no se completa (Tortora y Derrickson, 2006).

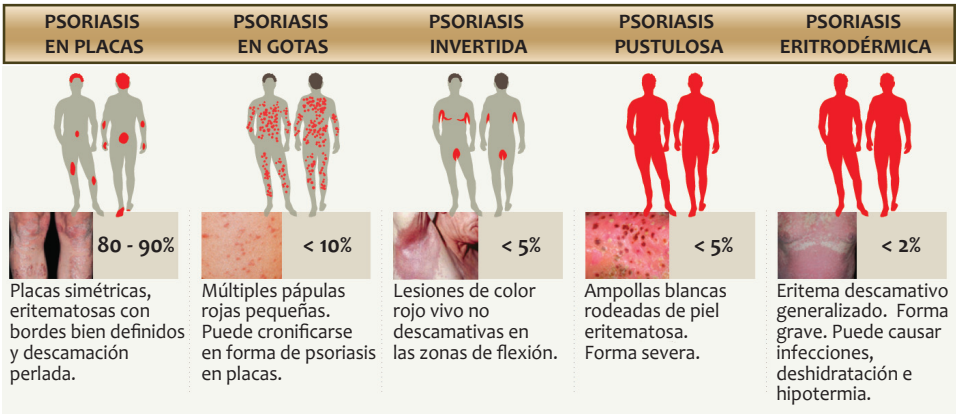
1.2 Dermis

Mientras que las células de la epidermis son numerosas e íntimamente asociadas, las de la dermis están separadas por un complejo conglomerado de material extracelular. Colágeno, elastina y otras fibras extracelulares son los principales constituyentes de la dermis, que dota de fuerza estructural y flexibilidad a la piel. Llenando el espacio entre el colágeno y la elastina se encuentran glicoproteínas como los glicosaminoglicanos y la fibronectina. Las células principales de la dermis son los fibroblastos, pero también existen células del sistema inmune, terminaciones nerviosas y vasos sanguíneos encargados de irrigar tanto la dermis como la epidermis (Figura 1).

2. Psoriasis

La psoriasis (del griego, picor) es una enfermedad inflamatoria, mediada por el sistema inmune y de afectación predominantemente cutánea, de curso crónico con exacerbaciones.

Pese a que existen varios tipos de psoriasis, algunos de ellos muy agresivos y potencialmente letales como las formas eritrodérmicas y las pustulosas (Figura 3), la manifestación más común es la denominada *psoriasis vulgaris* o en placas, que debuta con máculo-pápulas y placas eritemato-escamosas con borde perfectamente delimitado (Figuras 3 y 4). Suele tener disposición simétrica y las localizaciones más frecuentes son superficies de extensión (codos y rodillas), zona sacra y cuero cabelludo, siendo también común la afectación de las uñas. En la mayoría de los casos, estas lesiones son asintomáticas, aunque los pacientes suelen quejarse de prurito. Hasta en un 40% de los casos, dependiendo del estudio, se producen complicaciones como la artritis psoriásica, forma de curso más agresivo y altamente incapacitante en la que se ven afectadas las articulaciones (Cantini *y cols.*, 2010).



Adaptado de Crow (2012b)

Figura 3. La psoriasis es una enfermedad altamente heterogénea. Los pacientes suelen presentar un tipo de manifestaciones, aunque en ciertos casos pueden coexistir varios. Alrededor del 80% de los casos de psoriasis son diagnosticados como leves.

Figura 4. La psoriasis en placas es la forma más común.

La psoriasis es una patología de evolución imprevisible, con periodos libres de enfermedad y exacerbaciones de aparición y duración muy variables, pero en general su curso es crónico. Hasta el 80% de los pacientes la padecen durante toda su vida, ya sea de forma intermitente o continua. La mayoría de los casos, de carácter leve, no afectan a la salud general del paciente y son tratados en atención primaria. Sin embargo, alrededor del 20% de los pacientes sufren manifestaciones de moderadas a graves en las que es necesario un tratamiento sistémico. Además, la enfermedad tiende a agravarse con la edad y las formas más graves requieren de un cuidado intensivo de los afectados.

Se estima que entre un 2 y un 3% de la población mundial sufre psoriasis, con incidencias que van desde el 0,6 al 4,8%, dependiendo del clima y la herencia genética de la población (Naldi, 2004). Si bien puede aparecer a cualquier edad, sigue mayoritariamente una distribución bimodal con dos picos en la edad de inicio: entre los 15 y los 30 años y entre los 50 y los 60 (Perera *y cols.*, 2012). Afecta por igual a ambos sexos, aunque es más precoz en mujeres y personas con antecedentes familiares (Gelfand *y cols.*, 2005; Schon y Boehncke, 2005).

En España, se estima una prevalencia en torno al 1,4%, lo que corresponde a unas 470.000 – 570.000 personas, y una incidencia en aumento (Ferrandiz *y cols.*, 2001). Esto supone un coste anual de al menos 532 millones de euros para la sociedad española (Carrascosa *y cols.*, 2006). Además, la psoriasis se asocia con un mayor grado de comorbilidades, por lo que el coste final para los sistemas públicos de salud es equiparable al derivado de las enfermedades cardiovasculares, diabetes y enfermedades mentales (Perera *y cols.*, 2012).

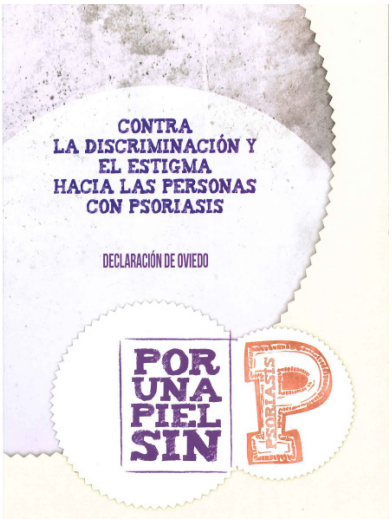


Figura 5. Diversas sociedades, como la Academia Española de Dermatología y Venereología, promueven acciones de información y concienciación social.

Pero más importantes que los costes económicos son los costes en salud y pérdida de calidad de vida. La psoriasis tiene un importante impacto sobre el individuo que la padece, abarcando desde aspectos físicos y psicológicos hasta aspectos sociales y ocupacionales. La inflamación sistémica crónica tiene un papel patogénico que deriva en una mayor incidencia de eventos cardiovasculares (Lu *y cols.*, 2013; Davidovici *y cols.*, 2010; Kaye *y cols.*, 2008), síndrome metabólico, dislipemia, diabetes, aterosclerosis, infarto de miocardio y linfomas (Revisado en Gottlieb y Dann, 2009). Además, el estigma estético que suponen las lesiones cutáneas conlleva una carga psicológica adicional, lo que se traduce en un mayor índice de depresión, ansiedad, obesidad, tabaquismo e ideación suicida (Figura 5) (Basavaraj *y cols.*, 2011; Kurd *y cols.*, 2010; Herron *y cols.*, 2005). Globalmente, varios estudios relacionan la gravedad de la patología con un aumento de la mortalidad en pacientes psoriásicos con respecto a la población general, produciéndose un acortamiento en la esperanza de vida en una media de 4 años (Abuabara *y cols.*, 2010; Gelfand *y cols.*, 2007).

2.1 Fenotipo y características histopatológicas

Clínicamente, la psoriasis en placas se caracteriza por la presencia de placas rojas de entre 1 y 10 cm^2 con escamas blancas o plateadas y que están claramente demarcadas de la piel adyacente (Figura 4). Estas placas pueden cubrir superficies variables del cuerpo, en función de la gravedad. Por ello, en un paciente psoriásico suelen convivir áreas de piel afectada (piel lesional) y áreas de piel sana (piel no lesional). La gravedad de la enfermedad se valora con una escala objetiva conocida como el índice de intensidad y gravedad de la psoriasis (Psoriasis Area and Severity Index, PASI) que

valora el eritema, la induración y la descamación de las lesiones en diferentes zonas del cuerpo y las relaciona con el área afectada en cada una de ellas.

Histológicamente, la psoriasis tiene una apariencia distintiva. Existe un marcado engrosamiento de la epidermis (acantosis) y una elongación de los pliegues epidérmicos (prolongaciones de la epidermis en la dermis) debido a la alta tasa de división de los queratinocitos. La diferenciación de los queratinocitos está muy alterada, mimetizando la "maduración regenerativa", un programa alternativo de diferenciación celular transitorio que se expresa durante la curación de heridas y que cursa con la inducción de la expresión de las citoqueratinas (CK) 6, 16 y 17. Por ello, muchos investigadores consideran las lesiones psoriásicas como un proceso constante de curación de heridas (Lowes *y cols.*, 2007).

La descamación superficial se debe a una maduración aberrante de los queratinocitos por efecto de los mediadores proinflamatorios liberados. El estrato granuloso, donde normalmente comienza la diferenciación, está muy disminuido o ausente en las lesiones psoriásicas. En consecuencia, el estrato córneo se forma a partir de queratinocitos inmaduros que retienen el núcleo de manera anormal (paraqueratosis). La descamación y la consiguiente rotura de la barrera protectora son consecuencia del fracaso de los corneocitos psoriásicos de apilarse normalmente, secretar lípidos extracelulares y adherirse los unos a los otros (Lowes *y cols.*, 2007).

Otra característica distintiva de la psoriasis es la presencia de neutrófilos en el estrato córneo y de infiltrado mononuclear en la epidermis, detectables por inmunotinción. Adicionalmente, existe una marcada infiltración de leucocitos mononucleares (células T y dendríticas) en la dermis. Los capilares dérmicos aparecen dilatados e hipertrofiados debido a la liberación de factores angiogénicos, lo que causa el eritema visible en las lesiones psoriásicas. Los leucocitos acceden al parénquima cutáneo a través de los capilares hipertróficos aunque existe una población residente que puede expandirse (Lowes *y cols.*, 2007; Schon y Boehncke, 2005) (Figura 6).

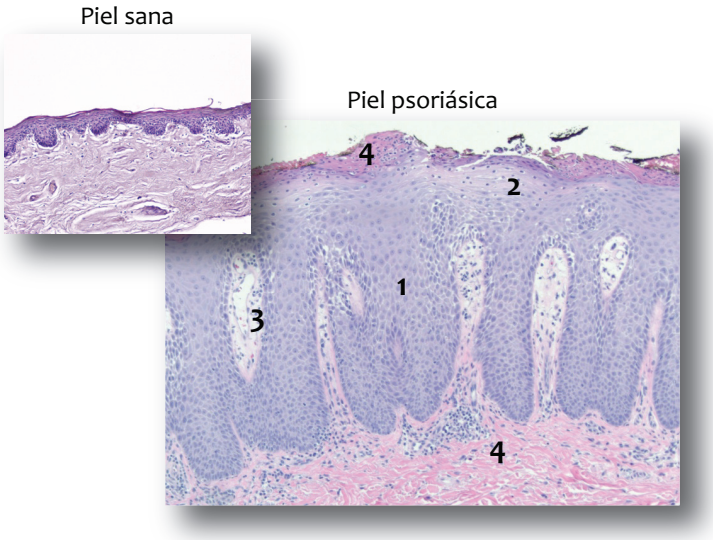


Figura 6. Los cambios histológicos característicos de la piel psoriásica incluyen: engrosamiento de la epidermis y elongación de los pliegues epidérmicos (acantosis) (1), ausencia del estrato granuloso y retención del núcleo en el estrato córneo (paraqueratosis) (2), dilatación e hipertrofia de los capilares dérmicos (3) y presencia de infiltrado inmunológico en dermis y epidermis (4).

2.2 Etiología

Aunque la causa de la pérdida de control en la proliferación de los queratinocitos es desconocida, numerosos estudios familiares han demostrado la existencia de una fuerte predisposición genética en pacientes psoriásicos. El 71% de los pacientes con psoriasis infantil tienen historia familiar y la concordancia en gemelos monocigóticos es del 70%, 20% en el caso de los dicigóticos. Sin embargo, la ausencia de una total concordancia en gemelos monocigóticos y la falta de un patrón claro de herencia sugiere una etiología multifactorial y una influencia de desencadenantes ambientales en el desarrollo de la enfermad (Capon *y cols.*, 2012; Perera *y cols.*, 2012; Schon y Boehncke, 2005).

2.2.1 Factores ambientales

Para muchos pacientes, los síntomas de la enfermedad mejoran durante el verano y empeoran en el invierno, apoyando la noción de que el curso de la enfermedad está influenciado por factores ambientales.

a) Infecciones

Numerosos estudios han demostrado la relación existente entre infecciones estreptocócicas del tracto respiratorio superior y la forma aguda de psoriasis en gotas. La incidencia previa de este tipo de infecciones llega hasta el 97% de los casos en pacientes que sufren este tipo de psoriasis, que aparece a una edad temprana y suele cronificarse en forma de psoriasis en placas (Roberson y Bowcock, 2010; Prinz, 2001). Además, este tipo de infecciones puede desencadenar exacerbaciones de la enfermedad (Valdimarsson *y cols.*, 2009). El mecanismo molecular postulado se basa en la homología existente entre la proteína M característica de estreptococos y las citoqueratinas CK16 y CK17, presentes en queratinocitos hiperproliferativos, que podrían actuar eventualmente como autoantígenos provocando la cronificación de la respuesta inmune (Elder *y cols.*, 2010; Roberson y Bowcock, 2010; Valdimarsson *y cols.*, 2009; Prinz, 2001).

b) Traumatismos físicos

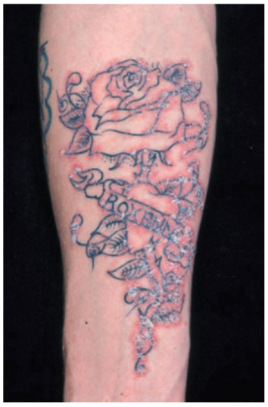


Figura 7. Fenómeno de Koebner.

Traumatismos físicos como heridas o tatuajes pueden desatar la aparición de lesiones psoriásicas en los lugares afectados (Fenómeno de Koebner), posiblemente a través de la liberación de mediadores proinflamatorios (Raychaudhuri *y cols.*, 2008), el desenmascaramiento de antígenos, o ambos (Perera *y cols.*, 2012; Schon y Boehncke, 2005). Además, las lesiones, así como la irritación, las quemaduras solares o las infecciones, inducen la expresión de las citoqueratinas CK6, CK16 y CK17 durante el proceso de regeneración de la piel, que conlleva un aumento en la tasa de división de los queratinocitos (Roberson y Bowcock, 2010).

c) Fármacos

Ciertas medicaciones analgésicas (antiinflamatorios no esteroideos) antidepresivas (litio), antihipertensivas (β-bloqueantes), antimaláricas y citocinas (Interferón (IFN)γ para el tratamiento de la hepatitis C o el melanoma maligno), pueden desencadenar tanto el inicio de la enfermedad como su agravamiento. Los mecanismos moleculares son poco conocidos en la mayoría de los casos (Perera *y cols.*, 2012; Schon y Boehncke, 2005).

d) Estilo de vida

El consumo de alcohol, el tabaquismo, el ejercicio físico y la obesidad, entre otros, pueden afectar la evolución de la enfermedad (Frankel *y cols.*, 2012; Armstrong *y cols.*, 2011; Davidovici *y cols.*, 2010; Liu *y cols.*, 2010a).

2.2.2 Factores genéticos

Estudios de ligamiento genético de genes candidatos, en familias afectadas por la enfermedad, condujeron al descubrimiento de 10 regiones de susceptibilidad para la psoriasis (PSORS) (Perera *y cols.*, 2012). Sin embargo, con la excepción de PSORS1, los resultados obtenidos no pudieron ser fehacientemente reproducidos (Elder *y cols.*, 2010). Hoy en día sabemos que se debe a la elevada frecuencia de los alelos de predisposición, presentes en muchas otras enfermedades genéticas complejas, así como en la población general, y a su menor importancia fenotípica, que en muchos casos está condicionada a la presencia de PSORS1 (Capon *y cols.*, 2012; Elder *y cols.*, 2010; Roberson y Bowcock, 2010).

PSORS1

Hasta el 60% de los pacientes psoriásicos son portadores del alelo *HLA-Cw*-0602* que codifica la proteína del complejo mayor de histocompatibilidad 1 (MHC-1) HLA-Cw6. Esta variación aumenta el riesgo de padecer la enfermedad 20 veces y está presente en el 10 - 15% de la población general. En los individuos homocigóticos, el riesgo aumenta 5 veces con respecto a los heterocigóticos (Perera *y cols.*, 2012).

Se ha observado que HLA-Cw6 presenta preferentemente péptidos comunes a las proteínas estreptocócicas M y a las queratinas presentes en queratinocitos hiperproliferativos CK16 y CK17. Este mecanismo es determinante para la psoriasis en gotas, que se inicia con una infección estreptocócica, es altamente dependiente de la presencia de esta mutación y puede cronificarse en forma de psoriasis en placas (Elder *y cols.*, 2010; Roberson y Bowcock, 2010; Valdimarsson *y cols.*, 2009; Prinz, 2001). De esta manera, se piensa que el alelo mutado *HLA-Cw*-0602* juega un papel fundamental en la pérdida de tolerancia inmunológica frente a autoantígenos de la piel (Elder *y cols.*, 2010), promoviendo una cronificación de la respuesta inmune.

Lugares de susceptibilidad no-MHC

Los estudios de asociación del genoma completo (GWAS), posibles gracias al avance en las técnicas de genotipado e identificación de polimorfismos de nucleótido simple (SNP) de alto rendimiento, están permitiendo el descubrimiento de nuevas regiones de susceptibilidad que mejoran el conocimiento de la inmunopatogénesis de la psoriasis (Ellinghaus *y cols.*, 2012; Elder *y cols.*, 2010; Strange *y cols.*, 2010; Stuart *y cols.*, 2010). Los últimos estudios postulan la existencia de 36 PSORS (Tsoi *y cols.*, 2012), que se resumen en la tabla 1.

Un estudio generalizado de los genes descubiertos nos revela una implicación clave de las vías de activación del factor de transcripción nuclear κB (NF-κB) y del eje T_H17, a través de la interleucina (IL)-23, así como del factor transductor de señal y activador de la transcripción 3 (STAT3).

Tabla 1 (Página siguiente). Resultados del más reciente metaanálisis de los locus asociados a susceptibilidad para la psoriasis. Se observa una implicación mayoritaria de las vías de señalización del NF-κB (en azul) y del eje IL-23/T_H17 (en rosa). Algunos genes intervienen en ambas vías (en morado). **Odds ratio** (razón de probabilidades): posibilidad de que una condición de salud o enfermedad se presente en un grupo de población frente al riesgo de que ocurra en otro. DC, células dendríticas; IFN, interferón; MHC-1, complejo mayor de histocompatibilidad 1; SNP, polimorfismos de nucleótido simple. Bibliografía: Capon *y cols.* (2012); Ellinghaus *y cols.* (2012); Perera *y cols.* (2012); Tsoi *y cols.* (2012); Bijlmakers *y cols.* (2011); Strange *y cols.* (2010); Stuart *y cols.* (2010); Capon *y cols.* (2008); Li *y cols.* (2006).

SNP	Cromosoma	Odds Ratio	Genes destacados	Descripción
Locus ya conocidos				
rs7552167	1	1.21	***IL28RA***	Señalización IFN
rs9988642	1	1.52	***IL23R***	Eje IL-23/T_H17
rs6677595	1	1.26	***LCE3B, LCE3D***	Diferenciación epidérmica
rs62149416	2	1.17	***REL***	Vía del NF-κB
rs17716942	2	1.27	***KCNH7, IFIH1***	Señalización IFN
rs27432	5	1.20	***ERAP1***	Procesamiento MHC-1
rs1295685	5	1.18	***IL13, IL4***	Diferenciación T_H2
rs2233278	5	1.59	***TNIP1***	Vía del NF-κB
rs12188300	5	1.58	***IL12B***	Diferenciación T_H1, Eje IL-23/T_H17
rs4406273	6	4.32	***HLA-B, HLA-C***	HLA-Cw6
rs33980500	6	1.52	***TRAF3IP2***	Vía del NF-κB/ Eje IL-23/TH17
rs582757	6	1.23	***TNFAIP3***	Vía del NF-κB
rs1250546	10	1.10	***ZMIZ1***	Vía TGF-β/ Smad
rs645078	11	1.09	***PRDX5***	Antioxidante
rs2066819	12	1.39	***STAT2, IL23A***	Eje IL-23/T_H17
rs8016947	14	1.16	***NFKBIA***	Vía del NF-κB
rs12445568	16	1.16	***FBXL19***	Vía del NF-κB
rs28998802	17	1.22	***NOS2***	Inmunidad innata, activación DC
rs34536443	19	1.88	***TYK2***	Eje IL-23/T_H17, señalización IFN
rs1056198	20	1.16	***RNF114***	Vía del NF-κB/ Eje IL-23/T_H17
rs4821124	22	1.13	***UBE2L3***	Ubiquitinización/ Vía del NF-κB
Nuevos locus identificados				
rs11121129	1	1.13	***TNFRSF9***	Generación células T $CD8^+$ de memoria
rs7536201	1	1.13	***RUNX3***	Diferenciación T_H1
rs10865331	2	1.12	***B3GNT2***	Hiperactivación linfocitos
rs9504361	6	1.12	***IRF4***	Eje IL-23/T_H17
rs2451258	6	1.12	***TAGAP***	Activación células T
rs2700987	7	1.11	***ELMO, DOCK2***	Señalización IFNα.
rs11795343	9	1.11	***DDX58***	Regula la producción de IFN de tipo I y II
rs10979182	9	1.12	***KLF4***	Activación macrófagos y T_H17
rs4561177	11	1.14	***ZC3H12C***	Activación macrófagos
rs3802826	11	1.12	***ETS1***	Regulador negativo T_H17
rs367569	16	1.13	***PRM3, SOCS1***	Eje IL-23/T_H17
rs963986	17	1.15	***STAT3, STAT5A/B***	Eje IL-23/T_H17
rs11652075	17	1.11	***CARD14***	Vía del NF-κB
rs545979	18	1.12	***POL1, MBD2***	Generación células T CD8+ de memoria
rs892085	19	1.17	***ILF3,CARM1***	Diferenciación células T. Vía del NF-κB

2.3 Inmunopatogénesis

La fisiopatología de la psoriasis es compleja y dinámica. Tanto células de la piel como del sistema inmune parecen estar implicadas en el establecimiento de un entorno proinflamatorio que impide el normal funcionamiento de la barrera epidérmica. Pese a los esfuerzos realizados en experimentación básica mediante el uso de animales de investigación y muestras de pacientes psoriásicos, así como arduos estudios genéticos, la información más fehaciente sobre los mecanismos inmunológicos subyacentes a la psoriasis se ha obtenido gracias a la clínica. Los resultados obtenidos mediante el uso de terapias biológicas en pacientes psoriásicos han obligado a los investigadores a reformular sus hipótesis en numerosas ocasiones.

2.3.1 Implicación de las células T

Inicialmente, se pensaba que la psoriasis era una enfermedad determinada por defectos en la diferenciación y proliferación de los queratinocitos, dadas las llamativas alteraciones visibles en los estudios histológicos. En este modelo, la inflamación era consecuencia de la alteración de la barrera epidérmica. Sin embargo, el descubrimiento fortuito de que la administración de agentes inmunosupresores, como la ciclosporina A, en pacientes con artritis reumatoide producía una sorprendente mejora en lesiones psoriásicas concomitantes desplazó el paradigma hacia las células del sistema inmune, especialmente los linfocitos T (Gottlieb *y cols.*, 1995; Ellis *y cols.*, 1986).

2.3.2 Papel del TNFα

La suerte volvió a favorecer a los investigadores y a revolucionar el estudio de la psoriasis cuando un paciente con enfermedad inflamatoria intestinal refractaria y psoriasis concomitante fue tratado con infliximab, un anticuerpo monoclonal anti-factor de necrosis tumoral α (TNFα) (Oh *y cols.*, 2000). Los buenos resultados

obtenidos llevaron a la aprobación de este tipo de terapias, que ya estaban siendo utilizadas en la artritis reumatoide, para el tratamiento de la psoriasis.

En la piel psoriásica, el TNFα es producido por queratinocitos, células dendríticas (DCs) y linfocitos T_H1, T_H17 y T_H22 (Eyerich *y cols.*, 2009; Lowes *y cols.*, 2008; Lowes *y cols.*, 2005). Una vez liberado, el TNFα señaliza a través de dos receptores: los receptores de TNF (TNFR) 1 y 2. TNFR-1 se encuentra en prácticamente la totalidad de células nucleadas mientras que TNFR-2 es inducible y se localiza preferentemente en células endoteliales y hematopoyéticas (Perera *y cols.*, 2012). La activación de los TNFRs inicia una cascada de eventos que conducen a la activación, entre otros, del NF-κB, que media los efectos proinflamatorios y antiapoptóticos del TNFα (Banno *y cols.*, 2005, 2004).

La eficacia de la terapia anti-TNFα se debe principalmente al efecto pleotrópico de esta citocina, que se pone de manifiesto en los estudios llevados a cabo en pacientes tratados con etanercept (Gottlieb *y cols.*, 2005). En estos estudios se ha visto que el bloqueo del efecto del TNFα conduce a la normalización en la proliferación y diferenciación de los queratinocitos, la disminución en los niveles de citocinas del eje T_H17, como IL-17, IL-22 y la quimiocina (C-C) ligando (CCL)20, y T_H1, como IFNγ. Además, el tratamiento con etanercept disminuye el infiltrado de DC y linfocitos. De esta manera, el bloqueo de la acción del TNFα interrumpe el ciclo vicioso de activación de DC, activación de células T y posterior liberación de mediadores proinflamatorios, clave en el mantenimiento de las lesiones psoriásicas (Perera *y cols.*, 2012).

2.3.3 Eje IL-23/T_H17

A la luz de los buenos resultados obtenidos con los tratamientos sistémicos inmunosupresores y anti-TNFα, la psoriasis pasó a considerarse una enfermedad inmune mediada por los linfocitos T_H1 y el TNFα. Sin embargo, esta teoría necesitaría

una nueva revisión después del descubrimiento de la IL-23 y de que los resultados experimentales y clínicos desviaran el foco hacia el eje IL-23/T_H17 en la patogénesis de la psoriasis (Lee *y cols.*, 2004).

El anticuerpo monoclonal anti-IL-12/IL-23 ustekinumab fue aprobado para el tratamiento de la psoriasis moderada y severa en base a los notables resultados obtenidos en dos ensayos clínicos de fase III (Leonardi *y cols.*, 2008; Papp *y cols.*, 2008) en el año 2009 (Perera *y cols.*, 2012). Diseñado a finales de los 90 con el objetivo de bloquear el efecto de la IL-12 (la IL-23 no se conocía todavía), ustekinumab bloquea la subunidad p40, común a la IL-12 y la IL-23 (Oppmann *y cols.*, 2000). De esta manera, impide la diferenciación de células T_H1, mediada por la IL-12, y T_H17 mediada por la IL-23, desarrollando una eficacia mayor incluso que la del etanercept (Reich *y cols.*, 2012). Estudios posteriores han demostrado que el efecto del ustekinumab se debe principalmente al bloqueo de la acción de la IL-23 (Lee *y cols.*, 2004).

La IL-23 es una citocina heterodimérica compuesta por una subunidad IL-23p19 característica y a una subunidad IL-12/23p40 que es común a la IL-12 (Oppmann *y cols.*, 2000). Está sobreexpresada en piel psoriásica, donde es producida predominantemente por DC y en menor medida por los queratinocitos (Piskin *y cols.*, 2006; Lee *y cols.*, 2004). Tras unirse a su receptor específico, la IL-23 ejerce sus efectos a través de la activación de la vía de señalización del STAT3 (Di Cesare *y cols.*, 2009), que será comentada posteriormente.

La importancia de la IL-23 en la psoriasis reside en su imprescindible papel en la diferenciación terminal de los linfocitos T_H17 (McGeachy *y cols.*, 2009; Langrish *y cols.*, 2005). La importancia de este subtipo de linfocitos T ha sido definitivamente confirmada mediante el uso en clínica de anticuerpos monoclonales anti-IL17, que en estudios preliminares han alcanzado una efectividad no conocida hasta la fecha (Martin *y cols.*, 2013; Leonardi *y cols.*, 2012; Papp *y cols.*, 2012b).

2.3.4 Visión actual

El actual modelo da un papel principal al eje IL-23/T_H17 en la patogénesis de la psoriasis. En él, la IL-23 secretada por las DC y los queratinocitos induce la activación de los linfocitos T_H17 que liberan IL-17A e IL-17F. Estas citocinas actúan sobre los queratinocitos dando lugar a una diferenciación aberrante y a la liberación de más IL-23, citocinas proinflamatorias (TNFα), quimiocinas (IL-8, CCL20, CCL27), péptidos antimicrobianos (LL-37, defensinas) y factores angiogénicos como el factor de crecimiento endotelial vascular (VEGF), que promueven el reclutamiento de más células del sistema inmune, dando lugar, de esta manera, a un ciclo de retroalimentación positiva que perpetua el estado inflamatorio crónico (Martin *y cols.*, 2013) (Figura 8).

En el actual modelo, se establecen, además, ciclos de retroalimentación positiva entre las células de la inmunidad innata y la adaptativa. Neutrófilos y mastocitos han sido identificados como la principal fuente de IL-17 en la piel psoriásica (Lin *y cols.*, 2011). Estos tipos celulares acuden al lugar de la lesión en las fases iniciales atraídos por las quimiocinas liberadas por los queratinocitos, principalmente IL-8. Allí son activadas a través de sus receptores tipo Toll (TLR) y contribuyen al mantenimiento del estado patológico mediante la liberación de esta citocina.

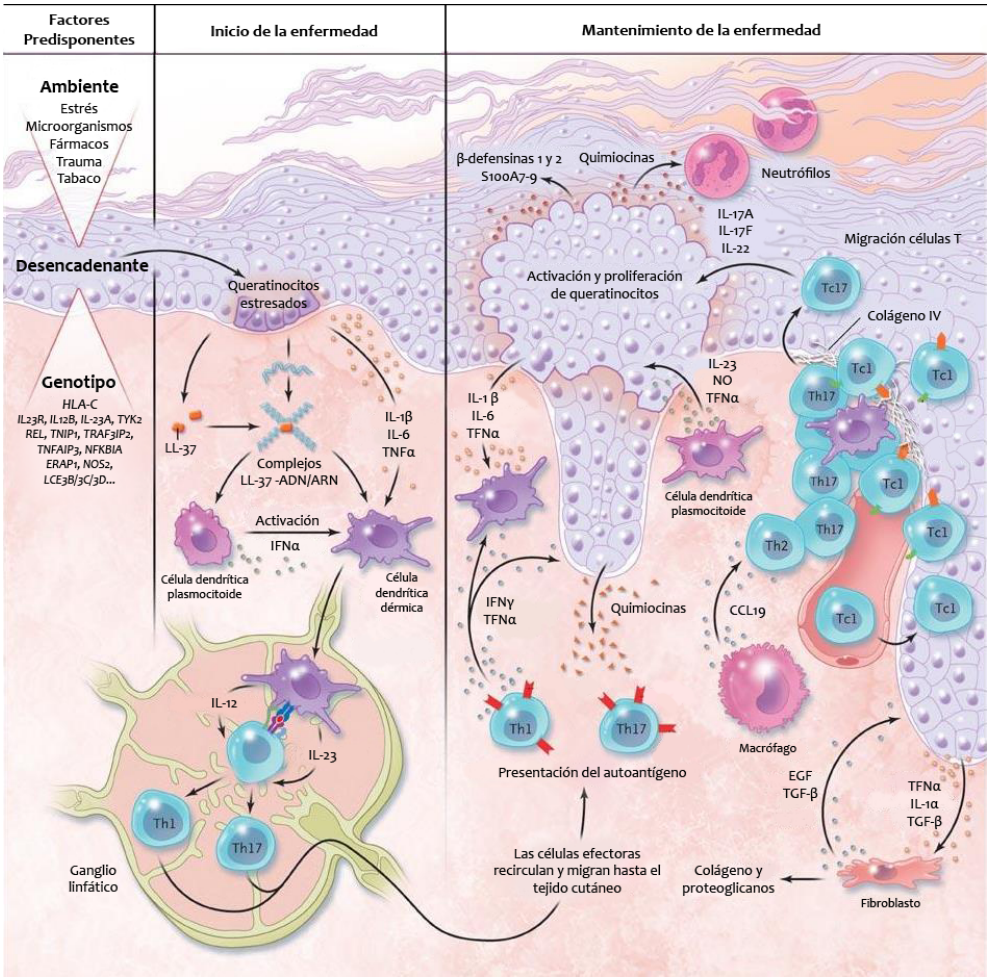
De especial interés para los investigadores es la observación de que, pese a que en todos los pacientes tratados con etanercept se produce una disminución de los mediadores de la respuesta inmune innata, la respuesta al tratamiento se da únicamente en aquellos pacientes en los que se normaliza la expresión de genes relacionados con la diferenciación y función de las células T_H17. Según lo observado, el TNFα favorece la diferenciación de monocitos a células dendríticas en presencia de células del estroma epidérmico (Chomarat *y cols.*, 2003). Por ello, el bloqueo de la acción del TNFα detiene el reclutamiento y diferenciación de células T_H17 por parte de las DC en lo que se considera uno de los pasos clave en los estadios iniciales de la

enfermedad (Zaba *y cols.*, 2009a; Zaba *y cols.*, 2009b; Bedini *y cols.*, 2007; Marble *y cols.*, 2007; Zaba *y cols.*, 2007). Además, se ha demostrado que el TNFα es capaz de potenciar los efectos de otras citocinas fundamentales del eje T_H17, como la IL-17 o la IL-22 (Chiricozzi *y cols.*, 2011; Eyerich *y cols.*, 2009).

Este modelo está en concordancia con los descubrimientos genéticos comentados en el apartado anterior, ya que la mayor parte de las mutaciones genéticas identificadas como de susceptibilidad para la psoriasis pertenecen a genes implicados en la vía de la IL-23 y del TNFα (Tabla 1). La presente tesis ahonda en el interés de estas dos vías mediante el estudio de los factores de transcripción que las orquestan: el NF-κB para el TNFα y el STAT3 para la IL-23, y de su posible explotación como dianas terapéuticas.

2.3.5 Inicio de la enfermedad

Lande *y cols.* (2007) realizaron una serie de experimentos en los que expusieron células dendríticas plasmocitoides (pDC) sanguíneas, que se sabía que jugaban un papel fundamental en las etapas iniciales de la formación de las placas psoriásicas, a extractos de piel sana y piel psoriásica. En estas condiciones, los extractos de piel psoriásica, a diferencia de los procedentes de piel normal, indujeron la liberación de IFNα por parte de las pDC. La profundización en este descubrimiento identificó el péptido antimicrobiano LL-37 como el agente instigador de la respuesta autoinmune al conjugarse con el ADN y ARN propio, liberado por queratinocitos dañados, y formar macrocomplejos capaces de activar a las pDC, así como a las células dendríticas mieloides, a través de los receptores TLR 7, 8 y 9 (Ganguly *y cols.*, 2009; Lande *y cols.*, 2007). La estimulación inicial de estos subtipos de DC induce la presentación de antígenos y la liberación de IFNα, TNFα, IL-6,IL-12 e IL-23 (Perera *y cols.*, 2012), que, junto a los mediadores liberados por los queratinocitos dañados, sientan las bases para la formación de la nueva placa psoriásica (Figura 8).



Adaptado de Nestle y cols. (2009)

Figura 8. Inmunopatogénesis de la psoriasis. En individuos con predisposición genética, un agente desencadenante activa los mecanismos inmunológicos de la respuesta inmune innata y adaptativa. Los queratinocitos estresados liberan IL-1β, IL-6, TNFα y péptidos antimicrobianos (LL-37) activando a otros queratinocitos y a las células dendríticas dérmicas (DCs). El daño puede causar la muerte de algunos queratinocitos lo que desencadena la liberación al medio extracelular de ácidos nucleicos. Las células dendríticas plasmocitoides (pDCs) acuden al lugar de la agresión, donde son estimuladas, junto con las células dendríticas residentes, por los macrocomplejos LL-37-ADN/ARN. La liberación de IFNα por las pDCs, junto a la estimulación de los receptores TLR por los macrocomplejos, permite la maduración de las células dendríticas mieloides dérmicas que viajan a los ganglios linfáticos donde presentan el autoantígeno a linfocitos T. La coestimulación con IL-12 e IL-23 polariza la respuesta inmune hacia T_H1 y T_H17, respectivamente. Los linfocitos maduros vuelven a la piel a través de los capilares dérmicos hipertróficos, estableciendo un ciclo de retroalimentación positiva entre DC inflamatorias, linfocitos y queratinocitos que perpetua el estado inflamatorio e impide la normal proliferación epidérmica. NO, óxido nítrico.

2.4 Farmacoterapia de la psoriasis

A día de hoy, no se dispone de una cura definitiva para la psoriasis. Por ello, la finalidad de los tratamientos antipsoriásicos es la de "limpiar" o "aclarar" las lesiones cutáneas y prevenir los nuevos brotes durante el mayor tiempo posible. Aun así, los últimos años han sido testigos de una auténtica revolución en la farmacoterapia de la psoriasis.

El uso de agentes biológicos ha permitido el tratamiento efectivo de las formas más severas de la enfermedad, antes tratadas con fármacos inmunosupresores obtenidos de la terapia para el trasplante de órganos, como la ciclosporina A o el metotrexato. Otros tratamientos, como los retinoides o la fototerapia, completaban un limitado abanico de opciones terapéuticas, de elevada toxicidad y eficacia moderada, haciendo muy difícil la vida de los pacientes más graves (Figura 9).

En la actualidad, disponemos de cinco agentes biológicos aprobados para el tratamiento de la psoriasis y otros tantos en las fases más avanzadas de los ensayos clínicos precomercialización. Los nuevos agentes anti-IL-17, brodalumab, ixekizumab

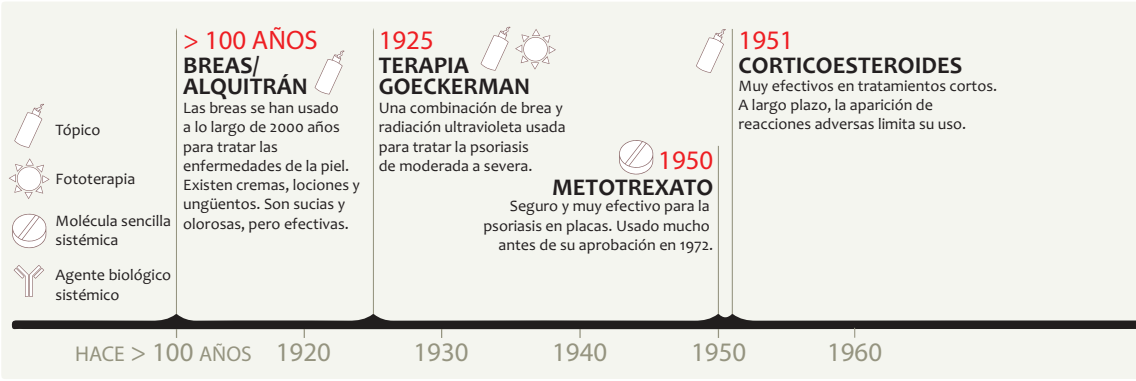
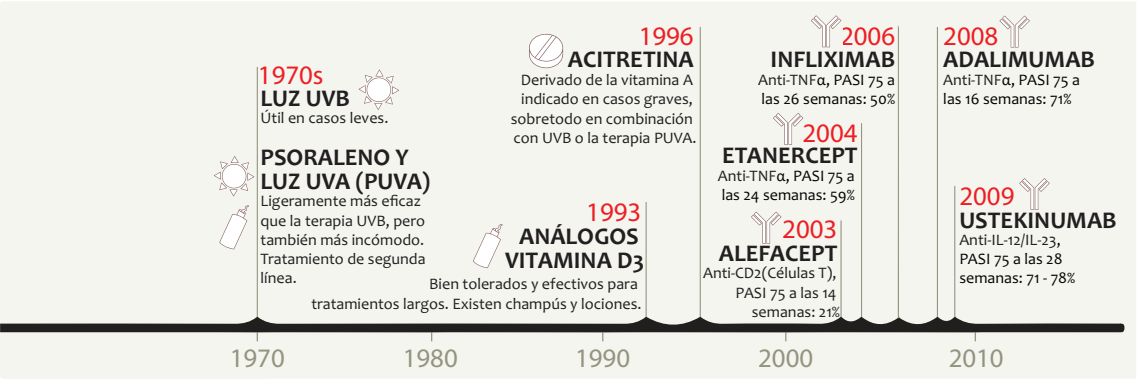


Figura 9. Evolución histórica de la farmacoterapia de la psoriasis. Un amplio espectro de terapias nuevas terapias biológicas son evaluadas en cuanto al marcador 'PASI 75', que representa el porcentaje

y secukinumab, que se encuentran actualmente en ensayos clínicos de fase III, han levantado grandes expectativas en la comunidad científica al alcanzar un PASI 100, es decir, una remisión completa de las lesiones psoriásicas, en la mitad de los pacientes a los que fueron administrados durante la fase II de los ensayos (Leonardi *y cols.*, 2012; Papp *y cols.*, 2012b; Rich *y cols.*, 2012).

Sin embargo, la mayoría de los pacientes psoriásicos no se beneficiarán de estos nuevos tratamientos, al menos a corto plazo. El elevado coste de las terapias biológicas, la vía de administración parenteral y el riesgo de aparición de reacciones adversas graves limita el uso de este tipo de agentes en los casos de leves a moderados, que suponen el 90% de los pacientes psoriásicos. Pese a los grandes avances logrados en las terapias sistémicas, los tratamientos tópicos empleados rutinariamente han permanecido invariables durante los últimos 20 años. Por ello, la búsqueda de nuevas moléculas sencillas, aptas para el tratamiento de las formas menos agresivas de la enfermedad, en administración oral o preferentemente tópica, supone un área de especial interés dentro de la actual farmacoterapia de la psoriasis.



Adaptado de Crow (2012b)

están disponibles para el tratamiento de la psoriasis, dependiendo de la severidad de la enfermedad. Las de pacientes en los que, como consecuencia del tratamiento, se reduce el PASI en al menos un 75%.

3. Vía de señalización del NF-κB

El factor nuclear potenciador de las cadenas ligeras κ de las células B activadas (NF-κB) es un factor de transcripción clave para la protección de la integridad de los organismos multicelulares frente a todo tipo de infecciones, pero también frente a agentes químicos dañinos, radiaciones y otras amenazas. Su importancia radica en su presencia en la práctica totalidad de las células animales, desde donde se alza como uno de los principales directores no sólo de la respuesta inmune innata, sino también de la respuesta inmune adaptativa.

3.1 Vía de señalización clásica

El NF-κB no es un factor de transcripción unitario sino que está constituido por una extensa familia de proteínas que interaccionan entre ellas para inducir la transcripción selectiva de cientos de genes mediante mecanismos que dependen de la participación de numerosos intermediarios. Pese a la complejidad del sistema, la vía de señalización mediada por las subunidades p65 y p50 es la que ejerce un mayor control del proceso inflamatorio (Ghosh y Hayden, 2012; Hayden y Ghosh, 2008).

La transcripción de los genes que codifican las subunidades del NF-κB se da incluso durante los estados latentes. De esta manera, en ausencia de estímulo activador, existe en el citoplasma una gran abundancia de las subunidades p65 y p50, que forman un pool latente de dímeros p65-p50 unidos al inhibidor IκB.

Existe un gran número de estímulos activadores del NF-κB, que mediante la unión a sus receptores específicos, tanto en la superficie como en el interior celular, activan una amplia variedad de vías de transducción. Todas estas vías convergen en la activación del complejo de las IKK quinasas, constituido por las proteínas IKKα, IKKβ e IKKγ (NEMO), que fosforila al inhibidor IκB. La fosforilación de IκB desencadena su desacoplamiento de los dímeros p65-p50, su ubiquitinización y su

posterior degradación por el proteosoma 26S. De esta manera, los dímeros p65-p50 quedan libres y se translocan al núcleo, donde encontrarán numerosos lugares de unión al ADN, llamados lugares κB. La arquitectura particular de cada lugar es clave para el reclutamiento de cofactores específicos, que determinarán la expresión de genes concretos (Ghosh y Hayden, 2012) (Figura 10).

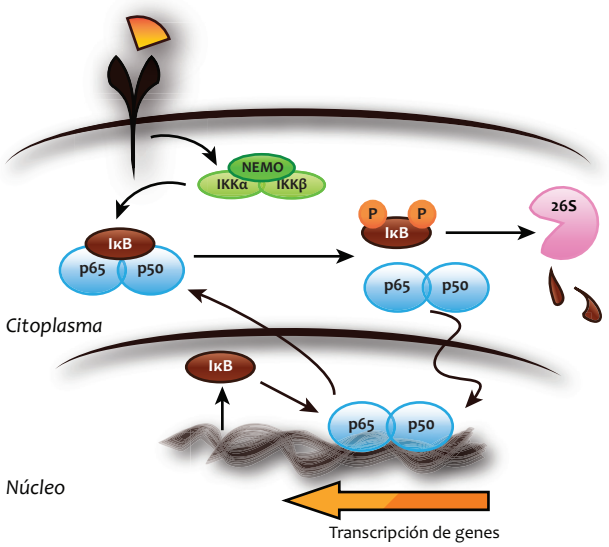


Figura 10. Vía de señalización del NF-κB. En su estado inactivo, los dímeros p65-p50 se encuentran en el citosol formando un complejo con el inhibidor IκB. A través de receptores integrales de membrana, una variedad de señales extracelulares activan la enzima cinasa (IKK) que fosforila al IκB. Esta modificación da lugar a la disociación del complejo IκB-NF-κB, y la degradación de IκB por el proteosoma 26S. Posteriormente, el dímero p65-p50 libre entra en el núcleo donde se une a secuencias de ADN específicas, iniciando la transcripción de numerosos genes entre los que se encuentra el IκB. El inhibidor recién formado es capaz de disociar los complejos NF-κB-ADN y devolver los dímeros p65-p50 al estado citosólico latente. De esta forma se vuelve al estado basal desde el que se comenzará un nuevo ciclo si el estímulo persiste.

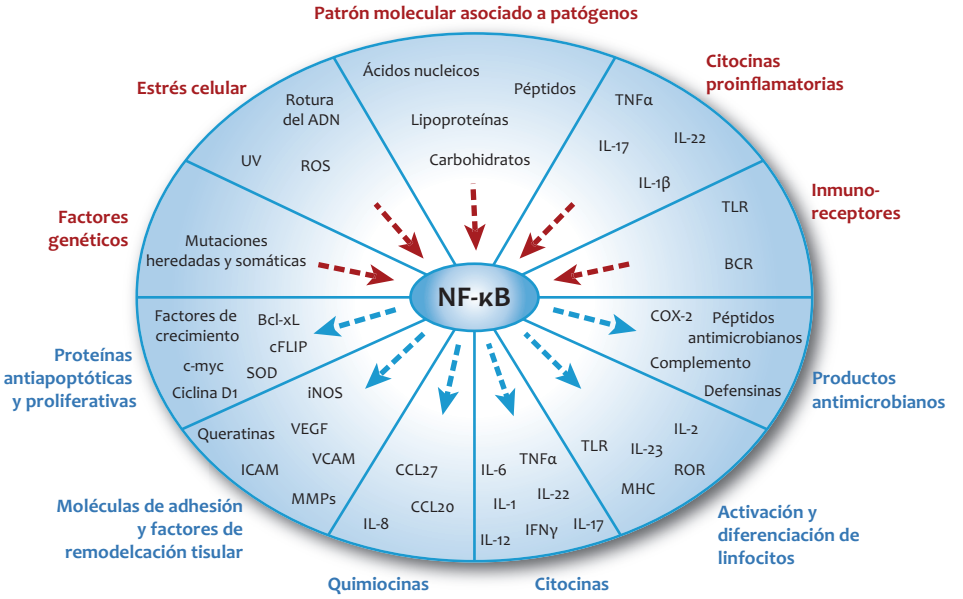
Durante este proceso las subunidades del NF-κB pueden sufrir modificaciones covalentes. Estos cambios suponen un punto de modulación adicional de la actividad transcripcional, ya que la transcripción de determinados genes depende directamente de su presencia o ausencia (Zhong *y cols.*, 2002). Entre estas modificaciones, cabe destacar la fosforilación de la subunidad p65, necesaria para la actividad transcripcional del NF-κB mediante el reclutamiento de los coactivadores CBP/p300 (Hayden y Ghosh, 2008).

3.2 NF-κB e inflamación

El NF-κB integra las señales de diversos estímulos proinflamatorios, promoviendo la liberación de mediadores capaces de propagar las señales de alerta, la síntesis de moléculas efectoras en la eliminación del agente invasor y la reparación de los posibles daños acaecidos. De esta manera, juega un papel fundamental en todas las etapas del proceso inflamatorio, siendo su principal director (Figura 11).

Cuando se produce una agresión, las células del estroma del tejido dañado o las células inmunes residentes, como los mastocitos o las células dendríticas, inician la respuesta inflamatoria activando vías mediadas por el NF-κB. Estas vías inducen la expresión de moléculas de adhesión y quimiocinas que conducen al reclutamiento de células de la respuesta inmune innata, como son los neutrófilos o los macrófagos. En estas células, el NF-κB es esencial para la producción de moléculas efectoras antimicrobianas y para la supervivencia en un entorno proinflamatorio. Finalmente, el NF-κB es necesario para la acción y la supervivencia de los linfocitos. Además, este factor nuclear tiene efectos antiapoptóticos y proliferativos, encaminados a la reparación del tejido dañado. De este modo, el NF-κB actúa en todas las fases de la inflamación, favoreciendo la respuesta inflamatoria en todos los tipos celulares, pero también protegiéndolos frente al daño que esta respuesta les causa.

Debido a su papel dual como potente inductora de la inflamación y como reguladora crítica de la supervivencia celular, la vía de activación del NF-κB está implicada en la patogénesis de varias enfermedades inflamatorias. De hecho, la pérdida de regulación de esta vía tiene importantes consecuencias patológicas y ha sido relacionada con cáncer, enfermedades inflamatorias y autoinmunes, shock séptico, infecciones virales o un desarrollo inmune inadecuado (Courtois y Gilmore, 2006).



Adaptado de (Ghosh y Hayden, 2012)

Figura 11. NF-κB como director de la inflamación. Señales diversas, que abarcan citocinas proinflamatorias, especies reactivas de oxígeno (ROS), luz ultravioleta (UV), roturas en el ADN o activación de receptores antigénicos, conducen a la activación del NF-κB, que induce la expresión de una gran variedad de genes. Estos programas transcripcionales tienen importantes consecuencias biológicas a través de la expresión de proteínas antiapoptóticas, factores de crecimiento, citocinas, quimiocinas y moduladores de la inmunidad innata y adaptativa.

3.3 NF-κB en psoriasis

Como eje articulador de la respuesta inflamatoria, el NF-κB está involucrado en la patogénesis de la psoriasis desde las fases más tempranas. Numerosos estímulos proinflamatorios como citocinas (TNFα, IL-1β, IL-17…), productos bacterianos y radicales libres generados en las placas psoriásicas, inician esta vía de señalización tanto en los queratinocitos como en las células de la inmunidad innata y adaptativa. De este modo, diversos estudios han demostrado un aumento de la expresión del NF-κB en piel psoriásica con respecto a piel normal (Lizzul *y cols.*, 2005; Westergaard *y cols.*, 2003). El NF-κB juega un papel principal tanto en la respuesta inflamatoria como en la regulación del ciclo celular de los queratinocitos a través de la inducción de genes

que codifican mediadores proinflamatorios, genes que amplifican y mantienen la inflamación crónica y genes antiapoptóticos (Hayden y Ghosh, 2008).

Además, como se ha comentado en apartados anteriores, los más recientes estudios genéticos han demostrado la relación existente entre alteraciones importantes en la biología del NF-κB y la susceptibilidad para padecer psoriasis (Tabla 1). El estudio de las variantes de susceptibilidad sugiere una deficiente regulación negativa de la acción proinflamatoria del NF-κB (Goldminz *y cols.*, 2013; Perera *y cols.*, 2012). Esta disfunción interrelaciona el estado alterado de los queratinocitos con el de las células inmunes, afectando a la proliferación celular, diferenciación y apoptosis, así como a la producción de citocinas y quimiocinas. De hecho, Rebholz *y cols.* (2007) demostraron que para producir un fenotipo psoriásico en ratón, era necesaria la sobreexpresión simultánea de NF-κB tanto en queratinocitos como en linfocitos, mientras que su sobreexpresión en sólo uno de estos tipos celulares era insuficiente para reproducir la enfermedad.

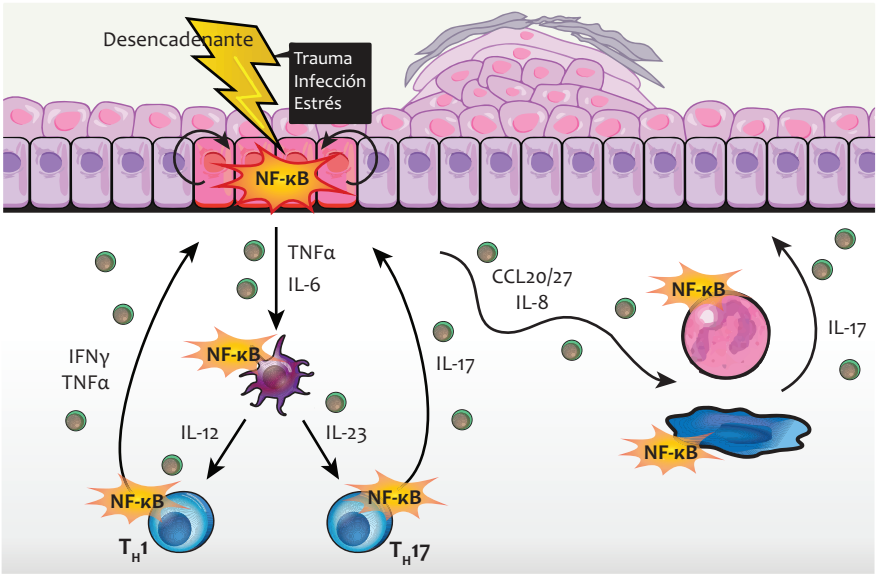
En consecuencia, numerosos tratamientos antipsoriásicos como tacrolimus, dimetilfumarato, calcipotriol, corticoesteroides o los agentes anti-TNFα actúan, en parte, disminuyendo los niveles de NF-κB (Perera *y cols.*, 2012; Gottlieb *y cols.*, 2005; Lizzul *y cols.*, 2005)

3.3.1 NF-κB en la respuesta inmune

Como se ha visto, el TNFα y el eje T_H17/IL-23 son cruciales en la patogénesis de la psoriasis. Pero, de hecho, muchas de las citocinas involucradas en ambas vías de activación, dependen también del NF-κB como principal factor transcripcional implicado.

En queratinocitos, el NF-κB es el principal factor de transcripción que media los efectos proinflamatorios y antiapoptóticos del TNFα, induciendo la síntesis de IL-1β, IL-6, IL-8 e IL-17C, entre otros muchos mediadores (Lowes *y cols.*, 2013; Johansen *y cols.*, 2011; Banno *y cols.*, 2005). Notablemente, entre las citocinas producidas se encuentra también el TNFα, el cual induce a su vez la activación del NF-κB, generándose un ciclo de retroalimentación positiva que mantiene y amplifica el proceso inflamatorio en las placas psoriásicas (Banno *y cols.*, 2004; Quivy y Van Lint, 2004). De hecho, se ha demostrado una relación directa entre los niveles de TNFα y la actividad NF-κB en pacientes psoriásicos, ya que el tratamiento con agentes bloqueantes del TNFα disminuye la activación de este factor nuclear y se correlaciona con la resolución de la enfermedad y la normalización en la epidermis (Gottlieb y cols., 2005; Lizzul y cols., 2005). En las células del sistema inmune, la disminución de TNFα conduce a la disminución de NF-κB, que no sólo detiene la producción adicional de TNFα sino que también elimina la producción paralela de citocinas y moléculas de adhesión.

La señalización mediante NF-κB tras la activación de los TLR es necesaria para la liberación de IL-6 e IL-23 por parte de queratinocitos y células dendríticas, citocinas necesarias para la diferenciación de los linfocitos T_H17 (Kuwabara *y cols.*, 2012; Ruan y Chen, 2012; Carmody *y cols.*, 2007). Además, este factor de transcripción está implicado en la liberación de IL-17A, IL-22, IFNγ y TNFα por linfocitos de pacientes psoriásicos (Kagami *y cols.*, 2010). Éstas citocinas, además, dependen del NF-κB para, entre otros efectos, inducir la expresión de las quimiocinas CCL20 y CCL27 en queratinocitos, contribuyendo al reclutamiento de más células del sistema inmune hacia la piel (Liu *y cols.*, 2010b; Vestergaard *y cols.*, 2005). Se postula que, mediante este mecanismo, las células T_H17 son capaces de promover su presencia estable en los tejidos psoriásicos (Harper *y cols.*, 2009)(Figura 12).



Adaptado de Goldminz y cols. (2013); Ainsworth (2012)

Figura 12. Papel central del NF-κB en la patogénesis de la psoriasis. La mayoría de las citocinas producidas durante el proceso psoriásico dependen de la activación del NF-κB. Además, el NF-κB es necesario para la activación y diferenciación de las células inmunes y su mantenimiento en el foco inflamatorio.

3.3.2 NF-κB en el control del ciclo celular de los queratinocitos

Pese al consenso existente en cuanto al papel proinflamatorio de la activación del NF-κB en los queratinocitos psoriásicos, existen todavía muchas incógnitas en cuanto a las consecuencias de su sobreactivación en el ciclo celular y engrosamiento epidérmico. En piel normal, el NF-κB se encuentra en el citoplasma de las células de la capa basal y en el núcleo de las células de las capas suprabasales en las que los queratinocitos dejan de dividirse, se diferencian y mueren (Kaufman y Fuchs, 2000; Seitz y cols., 1998). Se piensa que el NF-κB juega un papel fundamental en el control de este proceso ya que, en animales transgénicos, la sobreactivación del NF-κB produce un adelgazamiento de la epidermis, mientras que el bloqueo del NF-κB resulta en una epidermis engrosada con una diferenciación incompleta en la que los queratinocitos no siguen el programa apoptótico característico de las capas suprabasales (Kaufman y

Fuchs, 2000). Paralelamente, se ha demostrado que la inducción de NF-κB produce senescencia y resistencia a la apoptosis en queratinocitos sanos (Bernard y cols., 2004).

Sin embargo, numerosos estudios han demostrado un aumento de la activación del NF-κB en la epidermis psoriásica con localización predominantemente nuclear a lo largo de todas las capas epidérmicas (Lizzul y cols., 2005; Westergaard y cols., 2003). La explicación de esta paradoja podría encontrarse en el complejo entramado de proteínas que modulan continuamente la actividad transcripcional del NF-κB y la activación simultánea de otros factores de transcripción con efectos proliferativos. Además, como se ha comentado, los individuos que sufren psoriasis suelen ser portadores de mutaciones que alteran la vía de señalización del NF-κB. Las hipótesis más actuales postulan que, en el contexto de las placas psoriásicas, en las que los efectos de numerosos factores de transcripción convergen para dar lugar a un fenotipo característico en pacientes con predisposición genética, se produce un desequilibrio entre los efectos antiproliferativos y antiapoptóticos del NF-κB. Como resultado, el NF-κB protege a los queratinocitos hiperproliferativos de los efectos de agentes proapoptóticos, como el TNFα, mediante la síntesis de proteínas antiapoptóticas cuya expresión se encuentra aumentada en la psoriasis (Goldminz *y cols.*, 2013; Yang *y cols.*, 2009; Banno *y cols.*, 2005; Lizzul *y cols.*, 2005; Kocak *y cols.*, 2003).

4. Vía de señalización Jak/STAT

El estudio del mecanismo mediante el cual las citocinas inducen la expresión génica condujo, hace 20 años, al descubrimiento de la vía de señalización de las Janus cinasas (Jak)/ transductores de señal y activadores de la transcripción (STAT) (Darnell y cols., 1994), proporcionando un modelo relativamente sencillo para la comunicación entre la membrana y el núcleo celular tras la unión de gran variedad de citocinas y factores de crecimiento con sus receptores específicos.

La familia de los STAT consta de 7 miembros que actúan como factores de transcripción para regular el crecimiento, la supervivencia y la división celular. De entre ellos, STAT1 y, sobretodo, STAT3 son de especial relevancia para la patogénesis de la psoriasis y, por lo tanto, objeto de estudio en la presente tesis.

La vía de señalización Jak/STAT se inicia con la activación los receptores de citocinas tipo 1 y 2. Este tipo de receptores carecen de un dominio de actividad cinasa intrínseco y, en su lugar, se asocian con las Jak. De este modo, la unión del ligando específico permite la dimerización de este tipo de receptores y la activación de las Jak asociadas. Se inicia, así, una cascada de fosforilaciones que conducen a la exposición, por parte del receptor, de los lugares de anclaje de los STAT (Levy y Darnell, 2002) (Figura 13).

En condiciones basales, los STAT se encuentran mayoritariamente en el citoplasma en forma de monómeros inactivos. La activación de las Jak conduce al reclutamiento de dos monómeros de STAT por parte de los receptores de citocinas. Al unirse al receptor, los monómeros de STAT son fosforilados por las Jak en un residuo de tirosina. Esta modificación desencadena la disociación del receptor y la dimerización de los monómeros de STAT. Finalmente, los dímeros recién formados translocan al núcleo, donde promoverán la expresión de un amplio abanico de genes que dependerá del tipo celular en el que se haya desencadenado la respuesta (Levy y Darnell, 2002).

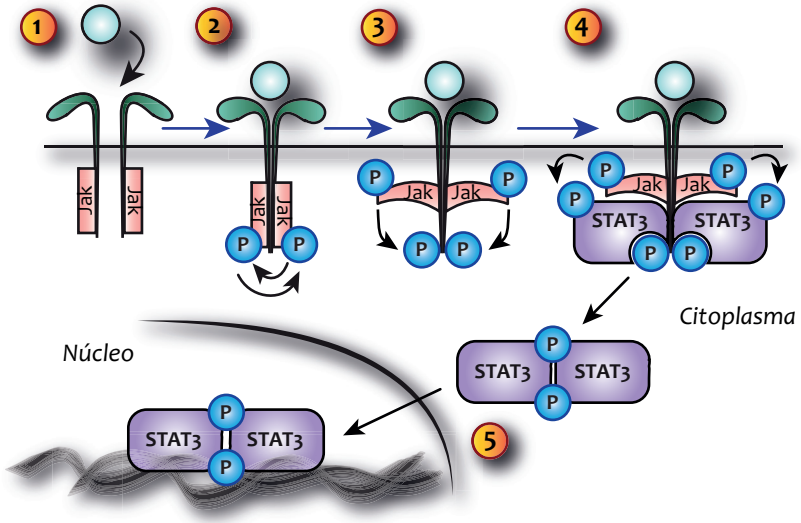


Figura 13. Vía de señalización Jak/STAT. La unión del ligando específico desencadena la dimerización del receptor (1), permitiendo la fosforilación recíproca de las Jak asociadas (2) y su activación. Como consecuencia, las Jak fosforilan al receptor (3) dando lugar al reclutamiento de dos monómeros de STAT, que son a su vez fosforilados (4), lo que permite su desacoplamiento del receptor y dimerización (5). El dímero de STAT recién formado es capaz de translocar al núcleo donde promoverá la expresión de genes específicos.

4.1 Regulación de la actividad transcripcional de los STAT

La actividad transcripcional de los STAT es regulada a través de modificaciones covalentes, entre las que destacan las fosforilaciones en residuos de tirosina y serina en el dominio de transactivación C terminal, la regulación del transporte nuclear y la unión a diversos supresores.

4.1.1 Fosforilación en el residuo tirosina

La fosforilación de un residuo de tirosina presente alrededor de la posición 700 supone un evento crítico para el inicio de la actividad transcripcional de los STAT, permitiendo su dimerización, translocación al núcleo y unión al ADN. Esta modificación es principalmente llevada a cabo por las Jak, aunque otras cinasas como la cinasa del receptor del factor de crecimiento epidérmico o las cinasas reguladas por

señales extracelulares (Extracellular signal-regulated kinases, ERK) pueden estar involucradas (Aggarwal *y cols.*, 2009).

La familia de cinasas asociadas a receptores Jak está compuesta por cuatro miembros: Jak1, Jak2, Jak3 y la tirosina cinasa 2 (Tyk2). Todas ellas están expresadas en la mayor parte de células excepto Jak3, que se encuentra mayoritariamente en células del sistema inmune (Schindler *y cols.*, 2007). Los principales sustratos de esta familia de cinasas son los STAT y una misma Jak puede fosforilar a varios de ellos. Cada receptor de citocinas se asocia preferentemente a una Jak, o a una combinación de ellas, aunque se desconocen las consecuencias de esta selectividad (Murray, 2007).

4.1.2 Fosforilación en el residuo serina

Entre las posiciones 720 y 730, los STAT poseen un residuo de serina que forma parte de un lugar consenso para las proteín cinasas activadas por mitógenos (Mitogen-activated protein kinase, MAPK). De este modo, numerosas cinasas como la proteína cinasa C (PKC), las MAPK, las ERK, las c-Jun N-terminal cinasas (JNK), la cinasa mTOR o cinasas dependientes de ciclina pueden fosforilar a los STAT en esta posición en respuesta a numerosos estímulos (Aggarwal *y cols.*, 2009; Schindler *y cols.*, 2007; Decker y Kovarik, 2000). La fosforilación de este residuo modula la actividad transcripcional de los STAT. Sin embargo, el efecto neto parece depender del estímulo y del contexto celular (Decker y Kovarik, 2000).

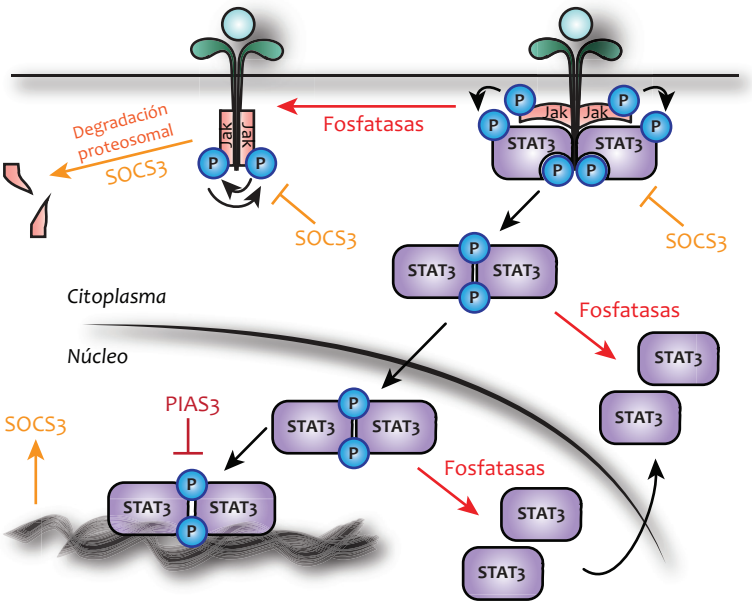
La importancia biológica de la fosforilación en el residuo de serina en la posición 727 (Ser727) de STAT3 es todavía objeto de debate (Reich, 2009; Decker y Kovarik, 2000). Algunos estudios la han relacionado con una localización mitocondrial de STAT3, donde aumenta la oxidación promoviendo la transformación celular por el oncogén Ras (Reich, 2009). Además, varios estudios han demostrado un incremento en los niveles de STAT3 fosforilado en Ser727 en las etapas tempranas de formación de diferentes tipos de tumores (Tkach *y cols.*, 2013; Aziz *y cols.*, 2010). Recientemente,

Sakaguchi *y cols.* (2012) han demostrado un aumento de la fosforilación en Ser727 en células de melanoma, donde provoca la translocación al núcleo de STAT3, confiriendo resistencia frente a la apoptosis, independientemente de la fosforilación en Tyr705.

4.1.3 Transporte nuclear y otros mecanismos de regulación

En condiciones basales, existe un equilibrio dinámico entre los niveles de STAT presentes en el núcleo y en el citoplasma. En el caso de STAT3, el paso a través de la membrana nuclear está mediado por las importinas α, β1 y Ran y parece ser independiente de la fosforilación en Tyr705 (Cimica *y cols.*, 2011; Liu *y cols.*, 2005).

La actividad transcripcional de los STAT está adicionalmente regulada por numerosos inhibidores. Entre ellos cabe destacar las proteínas supresoras de la señalización por citocinas (SOCS) por ser un producto de la activación de los STAT y formar un ciclo de retroalimentación negativa (Schindler *y cols.*, 2007). Otro ejemplo es la proteína inhibidora de STAT activado (PIAS) que actúa en el núcleo impidiendo la transcripción. Además, proteína fosfatasas, caspasas y degradación proteosomal dependiente de ubiquitinización también han sido identificados como moduladores de la actividad de los STAT (Schindler *y cols.*, 2007; Levy y Darnell, 2002). La pérdida de control de estos mecanismos está relacionada con numerosas patologías, especialmente cáncer (Aggarwal *y cols.*, 2009) (Figura 14).



Adaptado de (Lai y Johnson, 2010)

Figura 14. La vía de señalización de los STAT está regulada por diversas proteínas inhibidoras. Las SOCS pueden afectar a la unión de los STAT al receptor, así como a la actividad y la estabilidad proteica de las Jak. Las PIAS bloquea la transcripción. Finalmente, las fosfatasas devuelven al sistema a su estado basal.

4.2 STAT1

STAT1 es el principal factor de transcripción que media los efectos del IFNγ e IFNα, citocinas clave en el establecimiento de las placas psoriásicas (Perera y cols., 2012). Pese a su efecto antiproliferativo en queratinocitos, su importancia radica en su papel en la maduración de los linfocitos T_H1 (Oestreich y Weinmann, 2012). Varios estudios han demostrado un aumento en los niveles de STAT1 en piel psoriásica (Yao y cols., 2008; van der Fits y cols., 2004). Sin embargo, el nivel de activación de STAT1 en queratinocitos psoriásicos y los mecanismos moleculares subyacentes permanecen por discernir.

4.3 STAT3

Inicialmente identificado como un factor de transcripción dependiente de la IL-6 que promueve la expresión de genes de la fase aguda de la inflamación, STAT3 integra las respuestas de gran cantidad de mediadores entre los que se encuentran las citocinas de la familia de la IL-6 (IL-6, IL-11, IL-31…) y la IL-10 (IL-10, IL-20, IL-22, IL-23…), así como factores de crecimiento. Tras su activación, STAT3 promueve la expresión de genes que median la supervivencia (survivina, bclxl, mcl-1), la proliferación (c-fos, c-myc, cyclin D1), la invasión (metaloproteinasa de matriz 2), y la angiogénesis (VEGF) (Aggarwal *y cols.*, 2009; Schindler *y cols.*, 2007; Levy y Lee, 2002) (Figura 15). La importancia de este factor de transcripción para el desarrollo de los seres vivos se ha puesto de manifiesto mediante el desarrollo de ratones knock-out, que mueren a los 6 días de la embriogénesis (Takeda *y cols.*, 1997).

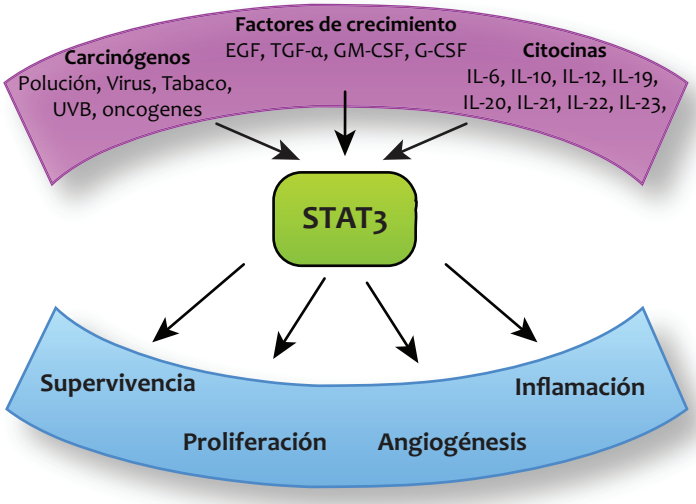


Figura 15. STAT3 integra las señales de diversos estímulos y coordina una respuesta celular que abarca las funciones celulares más elementales.

STAT3 en psoriasis

Sano *y cols.* (2005) pusieron de manifiesto la importancia de STAT3 en la patogénesis de la psoriasis mediante el uso de ratones transgénicos. La sobreexpresión localizada de STAT3 en los queratinocitos dio lugar a un fenotipo psoriásico capaz de reproducir rasgos característicos de la patología como son la hiperqueratosis, paraqueratosis, infiltrado en dermis y epidermis y dilatación vascular. Además, estos ratones eran reactivos al fenómeno de Koebner (Sano *y cols.*, 2005). Es de destacar que la piel trasplantada de estos ratones a ratones inmunodeficientes falló en reproducir el fenotipo psoriásico, poniendo de manifiesto la importancia de las interacciones entre queratinocitos activados y células del sistema inmune en la patogénesis de la psoriasis. Paralelamente, los autores de este estudio demostraron una sobreactivación de STAT3 en la piel lesional de paciente psoriásicos con respecto a la piel normal.

Posteriormente, se ha observado que muchas de las citocinas y factores de crecimiento aumentados en la psoriasis, como la IL-6, IL-20, IL-22 e IL-23 señalizan a través de la activación de STAT3 (Di Cesare *y cols.*, 2009; Sa *y cols.*, 2007; Zheng *y cols.*, 2007; Grossman *y cols.*, 1989). De este modo, se ha demostrado que STAT3 es necesario para la activación de linfocitos T_H1 y T_H17 por acción de la IL-2 y la IL-6, respectivamente (Akaishi y cols., 1998; Takeda y cols., 1998). Además, junto con NF-κB, STAT3 es necesario para la liberación de IL-17A, IL-22, IFNγ y TNFα por parte de los linfocitos T_H1 y T_H17 (Kagami *y cols.*, 2010; Cho *y cols.*, 2006). De hecho, personas con mutaciones en STAT3 desarrollan un cuadro severo de inmunodeficiencia debido a la incapacidad de generar linfocitos T_H17 (Ma *y cols.*, 2008; Milner *y cols.*, 2008). En queratinocitos, STAT3 media la acantosis inducida por IL-20, IL-22 e IL-23 (Sa *y cols.*, 2007; Zheng *y cols.*, 2007) y la expresión de CK17 tras la estimulación con IL-17A (Shi *y cols.*, 2011).

Toda esta evidencia ha dado lugar a la búsqueda de moléculas capaces de inhibir la activación de STAT3 para uso clínico. De hecho, este campo constituye en la

actualidad una de las fuentes más prometedoras de nuevos tratamientos para la psoriasis de leve a moderada (Crow, 2012a). Numerosos inhibidores de las Jak están siendo evaluados en ensayos clínicos y preclínicos (Punwani *y cols.*, 2012; Fridman *y cols.*, 2011; Chang *y cols.*, 2009). De entre ellos, cabe destacar Tofacitinib, una molécula desarrollada por Pfizer que se encuentra actualmente en ensayos clínicos de fase III (Papp *y cols.*, 2012a). Aunque no tan efectivos como las terapias biológicas, los buenos datos de seguridad y la posibilidad de administración oral y tópica respaldan su utilidad en los casos de psoriasis menos severa (Crow, 2012a).

5. Adenosina

La adenosina es un nucleósido formado por la unión de una base púrica (adenina) con un azúcar (ribosa) a través de un enlace β-N-glicosídico (Figura 16), implicado en diversas funciones bioquímicas, ya sea aisladamente o como constituyente de estructuras más complejas.

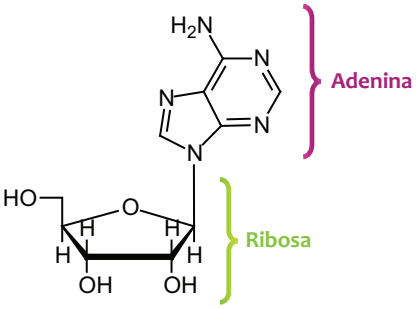


Figura 16. Estructura molecular de la adenosina.

Su papel fisiológico más conocido se debe a que forma la base de uno de los cuatro nucleótidos que constituyen el ADN y forma parte de diversas moléculas esenciales para el funcionamiento de las células como son el ATP, ADP, AMP, NADPH y FADH. Sin embargo, la adenosina juega también un papel fundamental en la regulación del metabolismo a través de la interacción con una serie de receptores de membrana.

5.1 Receptores de adenosina

Existen 4 subtipos de receptores de adenosina (A_1, $A2_A$, A_{2B} y A_3), todos ellos pertenecientes a la familia de receptores de 7 dominios transmembrana acoplados a proteínas G. En general, los receptores A_1 y A_3 se acoplan a proteínas Gi cuya estimulación produce la inhibición de la adenilato ciclasa, disminuyendo la concentración intracelular de adenosin monofosfato cíclico (AMPc), o la activación de la fosfolipasa C, que aumenta los niveles intracelulares de calcio. En contraposición, la estimulación de los receptores A_{2A} y A_{2B}, acoplados a proteínas Gs, induce a la adenilato ciclasa produciendo un aumento del AMPc (Hasko *y cols.*, 2008).

Los receptores A_1, A_{2A} y A_3 son de alta afinidad, lo que les permite ser activados por las concentraciones fisiológicas de adenosina, que se encuentran entre 30 y 300 nM

(Valls *y cols.*, 2009). Sin embargo, el receptor A_{2B} requiere de unos niveles de adenosina superiores a 10 μM. Por ello, la activación de este receptor se da únicamente en condiciones patológicas en las que los niveles de adenosina pueden alcanzar los 100 μM (Hasko *y cols.*, 2008; Haskó y Cronstein, 2004). Además de la concentración de adenosina, otros factores como la densidad de receptores o la funcionalidad de las vías de señalización intracelulares asociadas son también determinantes de la naturaleza y magnitud de las respuestas celulares (Hasko *y cols.*, 2008).

5.2 Adenosina extracelular

Los niveles extracelulares de adenosina resultan de su liberación por parte de las células y del catabolismo extracelular de nucleótidos de adenina. De este modo, la adenosina intracelular se genera por un aumento en el metabolismo del ATP durante el estrés celular y es liberada al medio extracelular mediante transportadores de nucleósidos equilibradores. Además, el ATP y el ADP extracelular son catabolizados por la acción secuencial de las ectoenzimas CD39 y CD73. CD39 hidroliza ATP y ADP a AMP mientras que CD73 defosforila el AMP a adenosina (Chen *y cols.*, 2013).

En contraposición, la adenosina generada a partir del ATP extracelular es rápidamente internalizada por las células. Allí es fosforilada por la adenosina cinasa para formar AMP o deaminada a inosina por acción de la adenosina deaminasa. Como consecuencia de la rápida internalización y metabolismo de la adenosina, los niveles de este mediador se mantienen bajos en tejidos sanos. Sin embargo, en condiciones patológicas el metabolismo de la adenosina no puede compensar su generación, lo que resulta en un marcado aumento en las concentraciones extracelulares (Hasko *y cols.*, 2008).

5.3 Agonistas y antagonistas de adenosina

El uso de herramientas farmacológicas ha sido de vital importancia para la identificación y diferenciación de los diferentes receptores de adenosina. En la presente tesis, el uso de agonistas y antagonistas selectivos nos ha permitido diseccionar el efecto global producido por la adenosina con el fin de facilitar su compresión.

De este modo, se ha empleado el 5-N-etil-carboxamidoadenosina (NECA) como agonista no selectivo de receptores A_{2A} y A_{2B} en combinación con dos antagonistas selectivos SCH 442426 (A_{2A}) y MRS 1706 (A_{2B}) con el fin de caracterizar las respuestas A_{2A} y A_{2B}. Para confirmar el efecto A_{2A}, se ha empleado adicionalmente el CGS 21680 (Tabla 2).

Ki (nM)	A_1	A_{2A}	A_{2B}	A_3
Adenosina	0,8	2300	18800 (EC_{50})	42
Agonistas				
NECA	14	20	2400	6,2
CGS 21680	290	27	88800	67
Antagonistas				
SCH 442416	1111	0,048	>10000	>10000
MRS 1706	157	112	1,39	230

Tabla 2. Afinidades de los agonistas y antagonistas de adenosina empleados en la presente tesis para los 4 subtipos de receptores en comparación con la adenosina. Ki, constante de disociación obtenida por estudios de binding por radioligando. Valores suministrados por Tocris Bioscience (R & D Systems).

5.4 Adenosina en psoriasis

Se ha demostrado que los niveles extracelulares de adenosina aumentan durante estados de isquemia, hipoxia, inflamación y trauma y que, en estas condiciones, las concentraciones son lo suficientemente elevadas para ejercer efectos inmunomoduladores (Haskó y Cronstein, 2004). Además, varios estudios han demostrado que la adenosina media el efecto antiinflamatorio de varios agentes antirreumáticos como el metotrexato, la sulfasalazina y los salicilatos, en varios modelos animales de inflamación aguda y crónica (Montesinos *y cols.*, 2000; Cronstein *y cols.*, 1999; Morabito *y cols.*, 1998; Cronstein *y cols.*, 1993).

El metotrexato es uno de los agentes inmunosupresores más prescritos por los dermatólogos para el tratamiento de una gran variedad de afecciones cutáneas. Desde 1951, el metotrexato se ha empleado para el tratamiento de la psoriasis de moderada a severa (Bangert y Costner, 2007). A pesar de la introducción de las nuevas y más efectivas terapias biológicas, es todavía considerado un agente de primera línea debido a su bajo coste, vía de administración oral y a la experiencia adquirida mediante años de uso en cuanto al manejo de su toxicidad (Warren *y cols.*, 2008).

El metotrexato es un análogo del ácido fólico, lo que le permite actuar como un antimetabolito para inhibir la proliferación celular. Sin embargo, en el tratamiento de enfermedades autoinmunes crónicas como la psoriasis, es común la adición de suplementos de ácido fólico con el fin de reducir la toxicidad de este compuesto, sin que este hecho suponga un compromiso de la acción antiinflamatoria (Bangert y Costner, 2007).

En base a estas observaciones, diversos estudios en modelos animales demostraron que el metotrexato promueve la acumulación extracelular de adenosina en los tejidos inflamados y que este mecanismo es esencial para su acción antiinflamatoria (Montesinos *y cols.*, 2000; Morabito *y cols.*, 1998; Cronstein *y cols.*, 1993). Además, se

observó que la ingesta de cafeína, que se comporta como antagonista no selectivo de la adenosina, puede disminuir la eficacia del tratamiento con metotrexato en pacientes con artritis reumatoide (Nesher *y cols.*, 2003). Por último, los polimorfismos que afectan a la vía de la adenosina en pacientes tratados con metotrexato son indicadores de la buena o mala respuesta al tratamiento (Wessels *y cols.*, 2006).

5.4.1 Efecto de la adenosina en queratinocitos

En la actualidad existe muy poca evidencia sobre el papel de la adenosina en los queratinocitos y los estudios son contradictorios. Por una parte, Brown *y cols.* (2000) demostraron la presencia del receptor A_{2B} y afirmaron que la adenosina induce un aumento del AMPc en queratinocitos humanos a través de la interacción con receptores de membrana, al ser bloqueado por el antagonista no selectivo teofilina. Sin embargo, la adenosina también ejerció un efecto antiproliferativo, que resultó ser independiente de la unión a estos receptores y parecía estar mediado por la internalización de este nucleósido.

Por otra parte, Braun *y cols.* (2006) detectaron en queratinocitos murinos la expresión de los subtipos de receptores tanto A_{2A} como A_{2B}. Además, en este estudio, la incubación con el agonista no selectivo NECA produjo un aumento en la proliferación de este tipo celular, aunque también parecían estar implicados otros receptores purinérgicos.

El papel beneficioso de la activación de la adenilato ciclasa en queratinocitos ha sido puesto de manifiesto en diversos estudios. De hecho, existe una molécula en ensayos clínicos, el apremilast, cuyo mecanismo de acción se basa en el aumento del AMPc intracelular que ha demostrado su eficacia con respecto al placebo. El efecto terapéutico está mediado por una reducción en la liberación de quimiocinas y la infiltración celular en lesiones cutáneas (Schafer, 2012).

A pesar de que la activación del receptor A_{2A} es capaz de inhibir citocinas proinflamatorias en sinoviocitos y otras células involucradas en procesos inflamatorios crónicos, muchos autores insisten en la posible variación de la respuesta en función del tejido o tipo celular y la necesidad de establecer el papel de la señalización de los receptores de adenosina en función de estos parámetros (Varani *y cols.*, 2010; Haskó *y cols.*, 2009). En la presente tesis se ha pretendido profundizar en el estudio del papel de los receptores de adenosina en la epidermis en relación con la vía de señalización del AMPc y la liberación de mediadores proinflamatorios en queratinocitos.

Objetivos

A pesar de los grandes avances alcanzados en la última década, la psoriasis sigue siendo una lacra tanto para los pacientes que la sufren como para los sistemas sanitarios de las sociedades desarrolladas. Por ello, tanto la iniciativa pública como privada han destinado gran cantidad de recursos a la investigación en este área, lo que ha permitido un tratamiento eficaz de las formas más severas de la enfermedad. Este gran esfuerzo se ha traducido en la aparición de cinco agentes biológicos nuevos en los últimos diez años, y tres más que se encuentran en las últimas fases de los ensayos clínicos.

Sin embargo, los tratamientos para las formas menos graves, que suponen el 90% de los casos de psoriasis, no han sido partícipes de esta revolución. De esta manera, la mayoría de pacientes psoriásicos siguen siendo tratados con un número reducido de opciones terapéuticas, que ha permanecido estancado durante años. De esta idea parte la motivación de la presente tesis, que ahonda en la búsqueda de nuevas moléculas sencillas aptas para el tratamiento de las formas menos agresivas de la enfermedad. Para ello, se ha establecido por primera vez en este laboratorio el cultivo de queratinocitos primarios humanos, así como modelos animales de psoriasis, con el objetivo de:

1) Profundizar en la comprensión de la respuesta fisiopatológica de los queratinocitos durante el proceso psoriásico con el objetivo de reconocer nuevas dianas terapéuticas
2) Buscar nuevas moléculas activas aplicables al tratamiento de esta enfermedad

Parte 1. Profundización en los mecanismos de la patología psoriásica

Pese al auge de los inhibidores de las Jak como nuevos agentes terapéuticos para el tratamiento de la psoriasis, poco se conoce sobre la activación de los STAT en la epidermis psoriásica. Por ello, el objetivo a desarrollar durante mi estancia en el laboratorio del profesor Knud Kragballe, en el Departamento de Dermatología de la Universidad de Aarhus (Dinamarca) fue el análisis de la expresión y activación de STAT3 en piel psoriásica lesional y no lesional, profundizando en los mecanismos moleculares que modulan este factor de transcripción en los queratinocitos. Los resultados obtenidos tanto en este estudio, como en otro, en el que participé en el análisis paralelo sobre el papel de STAT1 en piel psoriásica, han dado lugar a los dos artículos siguientes:

Artículo 1

Studies of Jak/STAT3 expression and signalling in psoriasis identifies STAT3-Ser727 phosphorylation as a modulator of transcriptional activity.

Andres RM, Hald A, Johansen C, Kragballe K, Iversen L. (2013) ***Experimental Dermatology*** (En prensa, DOI: 10.1111/exd.12128).

Artículo 2

STAT1 expression and activation is increased in lesional psoriatic skin.

Hald A, Andres RM, Salskov-Iversen ML, Kjellerup RB, Iversen L, Johansen C. (2013). ***British Journal of Dermatology*** 168, 302-10.

De igual manera, pese a que el metotrexato ha sido empleado durante más de 50 años para el tratamiento de la psoriasis, poco se sabe del efecto de la adenosina, mediador implicado en el mecanismo de acción de este fármaco en enfermedades inflamatorias, sobre queratinocitos primarios humanos. Por ello, en la presente tesis se ha caracterizado la expresión de los receptores de adenosina en epidermis normales y psoriásicas, así como las vías de señalización asociadas a su activación.

Artículo 3

Adenosine receptor expression is altered in psoriatic skin.
Andrés RM, Arasa J, Payá M, Navalón P, Valcuende F, Terencio MC, Montesinos MC. ***Letter to the Editor. Journal of Investigative Dermatology*** (En preparación).

Parte 2. Búsqueda de nuevos agentes antipsoriásicos

El segundo objetivo de la presente tesis ha sido la búsqueda de nuevas moléculas con potencial antiinflamatorio y antipsoriásico. Nuestro grupo de investigación tiene amplia experiencia en el estudio del efecto antiinflamatorio de compuestos de origen natural. El interés de este tipo de moléculas se basa en su gran diversidad y capacidad para interaccionar con sustratos biológicos frente a compuestos puramente sintéticos, desarrollada gracias al proceso de selección evolutiva al que son inherentemente sometidos.

La lógica del proceso de búsqueda de nuevos agentes terapéuticos establece su inicio en estudios de screening farmacológico en los que una batería de compuestos son testados en líneas celulares con el objetivo de seleccionar aquellos candidatos de

mayor potencial terapéutico. Siguiendo esta línea, en la presente tesis se han ensayado los siguientes compuestos de origen natural:

- Coscinolactamas A y B aisladas de la esponja marina *Coscinoderma mathewsi* por el laboratorio de la Dra. Maria Valeria D'Auria en el departamento de Química de las Sustancias Naturales de la Universidad de Nápoles, Italia.
- Pertamidas C y E aisladas de la esponja marina *Theonella swinhoei* por el laboratorio de la Dra. Maria Valeria D'Auria en el departamento de Química de las Sustancias Naturales de la Universidad de Nápoles, Italia.
- Derivados semisintéticos de Petrosaspongiolida M, sesterterpeno aislado de la esponja marina *Petrosaspongia nigra.* Estos derivados fueron sintetizados por el grupo de la Dra. Ines Bruno en el departamento de Química Farmacéutica de la Universidad de Salerno en Italia.
- Condroitín sulfato (CS), proteoglicano de origen animal ampliamente utilizado para el tratamiento de la osteoartritis, facilitado por la empresa Bioibérica S.A.

La conveniencia del uso de líneas celulares en los estadios iniciales de este tipo de estudios se basa en su facilidad de crecimiento y amplia disponibilidad, de gran utilidad cuando se trabaja con un gran número de moléculas. En este sentido, la línea de macrófagos murinos RAW 264.7 fue seleccionada por su potente y conocida respuesta inflamatoria ante diversos estímulos flogógenos. En esta línea celular, las moléculas en estudio fueron testadas respecto a su capacidad para inhibir mediadores generales de la inflamación, ampliamente producidos por los macrófagos, como son el óxido nítrico (NO) y la prostaglandina E_2 (PGE_2). Estos estudios preliminares dieron lugar a los siguientes artículos:

Artículo 4

Coscinolactams A and B: new nitrogen-containing sesterterpenoids from the marine sponge Coscinoderma mathewsi exerting anti-inflammatory properties.

De Marino S, Festa C, D'Auria MV, Bourguet-Kondracki M-L, Petek S, Debitus C, Andrés RM, Terencio MC, Payá M, Zampella A. (2009) ***Tetrahedron*** 65, 2905-9.

Artículo 5

Toward the Discovery of New Agents Able to Inhibit the Expression of Microsomal Prostaglandin E Synthase-1 Enzyme as Promising Tools in Drug Development.

De Simone R, Andrés RM, Aquino M, Bruno I, Guerrero MD, Terencio MC, Paya M, Riccio R. (2010) ***Chemical Biology & Drug Design*** 76, 17-24.

En una segunda fase, aquellas moléculas de perfil más interesante fueron ensayadas en cultivos primarios de queratinocitos humanos, con el fin de determinar su capacidad para inhibir la producción de TNFα e IL-8, dos citocinas proinflamatorias características de este tipo celular y de mayor relevancia para el proceso psoriásico. Pese a los buenos resultados obtenidos con todos ellos, se decidió continuar el estudio únicamente con el condroitín sulfato y el derivado 4-benzo[*b*]-tiofen-2-il-3-bromo-5-hidroxi-5H-furan-2-ona, (BTH), cabeza de serie de los derivados de petrosaspongiolida M ensayados en el artículo 5. Estos dos compuestos fueron estudiados en un amplio abanico de mediadores y factores de transcripción directamente implicados en la fisiopatología de los queratinocitos durante el proceso psoriásico, demostrando su potencial como nuevos agentes terapéuticos para el

tratamiento de esta enfermedad. Los resultados de estos estudios se recogen en los siguientes artículos:

Artículo 6

Perthamides C–F, potent human antipsoriatic cyclopeptides.
Festa C, De Marino S, Sepe V, D'Auria MV, Bifulco G, Andrés R, Terencio MC, Payá M, Debitus C, Zampella A. (2011) ***Tetrahedron*** 67, 7780-6.

Artículo 7

Potential antipsoriatic effect of chondroitin sulfate through inhibition of NF-kappaB and STAT3 in human keratinocytes.
Andres RM, Paya M, Montesinos MC, Ubeda A, Navalon P, Herrero M, Verges J, Terencio MC. (2013) ***Pharmacological Research*** 70, 20-6.

Artículo 8

NF-κB and STAT3 inhibition as therapeutic strategy in psoriasis: in vitro and in vivo effect of BTH.
Andrés RM, Montesinos MC, Navalón P, Payá M, Terencio MC. (2013) ***Journal of Investigative Dermatology*** (Aceptado para publicación).

Artículos científicos

Artículo 1

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Original Article

Studies of Jak/STAT3 expression and signalling in psoriasis identifies STAT3-Ser727 phosphorylation as a modulator of transcriptional activity

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Abstract: Jak/Tyk proteins have recently aroused as possible therapeutic targets for the treatment of psoriasis. In psoriasis, these proteins signal through STAT molecules including STAT3, and STAT3 expression and activation has been shown augmented in psoriatic lesions. Here, we characterized the expression of Jak/Tyk proteins in lesional compared with non-lesional psoriatic skin. Jak1, Jak2 mRNA and protein and Tyk2 mRNA appeared to be downregulated, whereas Jak3 mRNA expression was increased. Moreover, STAT3 expression and activation was examined in psoriasis. STAT3 is activated at two phosphorylation sites: Tyr705 and Ser727. Both phosphorylation sites were phosphorylated in lesional psoriatic skin. The activation of STAT3 by Jak/Tyk proteins was studied in cultured normal human keratinocytes. Tyr705 phosphorylation was induced by IL-6 and IL-20 in a Jak2-dependent manner, and moreover, phosphorylation of Tyr705 produced a strong increase in STAT3 transcriptional activity. TNFα, 12-O-Tetradecanoylphorbol 13-acetate (TPA) and UVB irradiation induced Ser727 phosphorylation of STAT3 in an ERK1/2- and p38 MAPK-dependent manner, which resulted in a modulatory effect on STAT3 transcriptional activity. Our results demonstrate how different signalling pathways can integrate and lead to regulation of STAT3 transcriptional activity.

Key words: IL-20 – IL-6 – Jak proteins – psoriasis – STAT3

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Introduction

The mammalian Janus kinase (Jak) family of non-receptor protein tyrosine kinases consists of four members – Jak1, 2 and 3 and tyrosine kinase 2 (Tyk2). The Jak/signal transducers and activators of transcription (STAT) pathway plays a critical role in mediating inflammatory immune responses by converting cytokine signals into genomic responses regulating proliferation and differentiation of the immune cells. A broad range of cytokines signal through the 'type I' and 'type II' cytokine receptors, which lack intrinsic intracellular tyrosine kinase domains. Instead, these receptors associate with Jak kinases for receptor phosphorylation, which creates a docking site for downstream signalling molecules, including STAT transcription factors (1,2). Once the STATs are recruited to the docking site, they become phosphorylated by the receptor-associated Jaks leading to STAT dissociation from the receptor complex, dimerization, activation, translocation to the nucleus and finally regulation of gene transcription (3).

Recently, Jak proteins have emerged as possible new therapeutic targets for the treatment of autoimmune inflammatory diseases, including psoriasis (4,5). Several Jak inhibitors are being tested in clinical and preclinical trials with promising results. A Jak1/2 inhibitor tested by topical administration *in vivo* suppressed STAT3 phosphorylation, oedema, lymphocyte infiltration, and keratinocyte proliferation in a murine contact hypersensitivity model and inhibited tissue inflammation induced by intradermal IL-23, with good tolerability (6). A small molecule inhibitor of Jak3 produced a marked attenuation of skin lesions following 6 weeks of treatment in the CD18 mutant PL/J mice T-cell-dependent psoriasis form model with decreased levels of inflammatory cytokines and epidermal T-cell infiltration (7). Moreover, Tofacitinib (CP-690,550), a Jak1/3 inhibitor and the leading drug candidate of this class for psoriasis treatment, is currently in phase III clinical testing. In a phase I study, it has proven to reduce the Psoriatic Lesion Severity Sum (PLSS) score by 72%, compared with 12% for placebo in a 2-week treatment and with only mild adverse events (8).

Several studies have implicated STAT3 in regulating fundamental cellular biological processes such as proliferation, differentiation, malignant transformation, survival and apoptosis (9). Total STAT3 expression has also been proven to be upregulated in psoriatic lesions and a transgenic mouse model constitutively overexpressing activated STAT3 in the basal stem-cell layer of the epidermis was able to reproduce many of the characteristic features of psoriasis (10,11). Moreover, several cytokines and growth factors upregulated in psoriasis such as IL-6 and the IL-20 family cytokines (IL-19, IL-20, IL-22 and IL-24) are able to induce STAT3 activation (11–13). In addition STAT3 has been shown to play a role in psoriasis-associated IL-23 signalling pathway (9).

STAT3 is activated by phosphorylation at two different phosphorylation sites: Tyrosine 705 (Tyr705) and Serine 727 (Ser727). Tyr705 phosphorylation is essential for STAT dimerization, nuclear translocation and DNA binding, whereas C-terminal Ser727 phosphorylation is required for maximal transcriptional activity (14). Phosphorylation of Tyr705 is mainly conducted by Jak proteins, although other receptor and non-receptor protein tyrosine kinases including the epidermal growth factor receptor (EGFR) kinase, Src and extracellular signal-regulated kinase (ERK) may be involved. In addition, numerous serine kinases have been

implicated in the phosphorylation of STAT3 at Ser727. These include protein kinase C (PKC), mitogen-activated protein kinases (MAPK), ERK and CDK5 (15,16).

The purpose of this study was to characterize Jak/Tyk and STAT3 expression and activation in lesional compared with non-lesional psoriatic skin, and to conduct functional studies on how STAT3 activation is regulated in human keratinocytes.

Methods

Biopsies

Informed written consent was obtained before biopsies were taken from lesional and non-lesional skin. Biopsies were taken as previously described (17). Briefly, keratome biopsies ($n = 5$) from lesional and non-lesional plaque-type psoriatic skin were taken from the centre of a plaque from patients with moderate to severe chronic stable plaque psoriasis (absolute PASI score between 10 and 18) from either the upper or lower extremities and used for protein detection, whereas 4-mm punch biopsies ($n = 10$) were taken for mRNA and immunofluorescence analysis. Biopsies were taken as paired samples, and biopsies from non-lesional psoriatic skin were taken from the same body region as biopsies from lesional psoriatic skin at a distance of at least 5 cm from a lesional plaque. Keratome biopsies and punch biopsies were taken from different patients.

The patients had received no topical treatment for at least 2 weeks and no systemic treatment for at least 4 weeks before the procedure. The study was approved by the local ethical committee (Region Midtjylland) and carried out according to the Declaration of Helsinki Principles.

Cell cultures

Normal adult human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery as previously described (18). Second passage keratinocytes were grown in serum-free keratinocyte medium supplemented with human recombinant epidermal growth factor, bovine pituitary extract, and gentamicin (all from Gibco/Invitrogen Carlsbad, CA, USA). Cells were grown at 37°C and 5% CO_2 in a humidified incubator. Fresh medium was added every second day. By the time the cells achieved 60–80% confluency, the medium was changed to growth factor–free keratinocyte medium, and the cells were incubated for another 24 h before stimulation.

Cells were stimulated with either IL-6 (50 ng/ml), IL-20 (10 nM), TNFα (10 ng/ml), TPA (100 nM), UVB (250 J/m^2) or a combination of these stimuli. Vehicle (phosphate-buffered saline with 0.15% BSA) was added to control. Cytokines were purchased from R&D Systems (Abingdon, UK). The UV source was a linear bank of UVB fluorescence tubes emitting UVB light (Phillips TL12).

In some experiments, keratinocytes were pretreated for 45 min with a Jak2 Inhibitor '1,2,3,4,5,6-Hexabromocyclohexane' (50 μM), a p38 MAPK inhibitor 'SB202190' (10 μM) or an ERK1/2 inhibitor 'PD98059' (50 μM) all of them from Calbiochem, San Diego, CA, USA. Controls contained an equal volume of DMSO (1% vol/vol).

Quantitative PCR

RNA isolation from skin biopsies, reverse transcription and quantitative RT-PCR were performed as previously described (19). In brief, SV Total RNA Isolation System (Promega, Madison, WI, USA) and Taqman Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) were used according to the manufacturer's protocol for construction of complimentary DNA including the use of random hexamers as primers.

The mRNA expression of STAT3, Jak1, Jak2, Jak3, Tyk2 and the housekeeping gene *RPLP0* was determined using Taqman 20x Assays-On-Demand (FAM-labeled MGB-probes) gene expression assay mix (Applied Biosystems; assay ID: Hs01047580_m1, Hs01 026983_m1, Hs01078124_m1, Hs00169663_m1, Hs00177464_m1 and Hs99999902_m1, respectively) and qPCR Supermix-UDG (Invitrogen). All samples were analysed in triplets on a StepOnePlus machine (Applied Biosystems), and the reactions were run as singleplex. Relative gene expression levels were determined using the relative standard curve method. Briefly, a standard curve for each gene was made of fourfold serial dilutions of total RNA from punch biopsies from the skin of psoriatic patients. The curve was then used to calculate relative amounts of target mRNA in the samples.

Western blotting

Total cell protein was extracted from keratome biopsies as previously described (20). Briefly, biopsy specimens were homogenized in cell lysis buffer and incubated on ice for 30 min before centrifugation.

Cell cultures were washed in ice-cold phosphate-buffered saline and lysed in a buffer containing 50 mM Tris-HCL, pH 6.8, 10 mM dithiothreitol, 10 mM b-glycerophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 10% glycerol, 2.5% SDS added PMSF and a cocktail of proteinase inhibitors (Roche, Mannheim, Germany). The lysates were boiled for 3 min, incubated on ice with benzon nuclease for 30 min and finally centrifuged at 13 600 g for 3 min.

Supernatants were removed and protein concentrations were determined by Bradford assay. The samples were stored at −80°C until further use. Equal amounts of protein were separated on a NuPAGE 4–12% Bis-Tris gel (Invitrogen) and blotted onto nitrocellulose membranes.

Membranes were incubated with primary antibodies and detected by horseradish peroxidase conjugated secondary antibodies in a standard ECL reaction (GE Healthcare, Wessling, Germany). Primary anti-STAT3, anti-phospho-STAT3 (Tyr705), anti-phospho-STAT3 (Ser727), anti-Jak1, anti-Jak2, anti-Jak3 and anti-Tyk2 antibodies, and secondary anti-rabbit antibody were from Cell Signaling Technology, Beverly, MA, USA. As a protein loading control, all the bands were normalized to β-actin. Band intensity was analysed using Kodak 1D Image Analysis software (Kodak, Rochester, NY, USA).

Immunofluorescence analysis

Four-μm paraffin-embedded sections of lesional and non-lesional psoriatic skin were deparaffinized and rehydrated. Antigen retrieval was performed by boiling in TEG buffer (pH 9) in a microwave. Blockage of the non-specific antibody binding sites was achieved by incubating the samples for 30 min with Image-iT ™ FX Signal Enhancer (Invitrogen).

Sections were then incubated overnight at 4°C with rabbit anti-phospho-STAT3 (Tyr705; 1:25) or rabbit anti-phospho-STAT3 (Ser727; 1:100) form Cell Signaling Technology, diluted in TBS buffer with 5% goat serum. Secondary staining was obtained incubating with Alexa Fluor® 488 Dye (Invitrogen; 1:300) diluted in TBS buffer with 5% goat serum for 1 h. Finally, the samples were washed and nuclear staining was performed by embedding samples in Prolong Gold antifade reagent with DAPI (Invitrogen).

Samples were evaluated by epifluorescence microscopy. As a negative control, sections were incubated with blocking buffer without primary antibody and as an isotype control with normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) instead of primary antibody.

Transfection and determination of promoter activity

STAT3 promoter studies were carried out according to manufacturer's instructions. Briefly, human keratinocytes were cultured in 24-well plates and transfected at 60–70% confluence. Cells were transfected with 0.5 μg of the STAT3-promoter-luciferase-Renilla reporter plasmids (SABiosciences, QIAGEN, Frederick, MD, USA) using Fugene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany). 24 h after transfection, cells were stimulated with IL-6 (50 ng/ml), IL-20 (10 nM), TNFα (10 ng/ml) or a combination of these stimuli for different time periods. After stimulation, cells were harvested with 100-μl passive lysis buffer from Promega, and Firefly and Renilla luciferase activity was measured by the Dual Luciferase assay system (Promega) on aFluoroskan Ascent Fl (BIE & Berntsen, Rodovre, Denmark). A positive control expressing both Firefly and Renilla luciferase and two negative controls, each one of them expressing only one of the luciferase activities were added to all the experiments. Promoter activity was reported as the ratio between Firefly and Renilla luciferase activities in each sample.

Statistical analysis

Results are presented as mean ± SEM. Statistical analyses were performed using one-way analysis of variance followed by Dunnett's *t*-test for multiple comparisons and two-tailed paired Student's *t*-test for dual comparisons. A probability of $P < 0.05$ was regarded as statistically significant.

Results

Jak and Tyk expression in psoriatic skin

Jak and Tyk expression was analysed in paired samples of lesional and non-lesional psoriatic skin by RT-PCR ($n = 10$) and Western blotting ($n = 5$).

Jak1 and Tyk2 mRNA expression was found to be significantly downregulated in lesional psoriatic skin compared with paired samples of non-lesional psoriatic skin. Jak3 mRNA expression, in contrast, was significantly increased, whereas Jak2 expression was not significantly modified at the mRNA level (Fig. 1a).

Jak1 and Jak2 protein levels showed also a tendency towards downregulation in lesional skin (Fig. 1b). This tendency was corroborated by the densitometric study of the bands, but it did not become significant in the five samples studied ($P = 0.0664$ and $P = 0.0703$, respectively; Figure S1a). Jak3 and Tyk2 Western blotting was also conducted but no specific signals were detected with the available antibodies (Figure S1b).

STAT3 expression and phosphorylation is increased in lesional psoriatic skin

STAT3 expression was studied in paired samples from lesional and non-lesional psoriatic skin by RT-PCR and Western blotting. RT-PCR results showed a significant 3-fold upregulation of STAT3 mRNA in lesional psoriatic skin compared with non-lesional skin ($n = 10$; Fig. 2a). This increase was also seen at the protein level, although densitometric analysis did not reveal a significant change ($n = 5$, $P = 0.0508$; Fig. 2b and S2a).

Studies of STAT3 activation were also conducted. STAT3 is activated through phosphorylation at two different phosphorylation sites: Tyr705 and Ser727. Densitometric analysis of the band intensity from the Western blotting revealed a significant upregulation ($P < 0.001$) in both phosphorylation sites in lesional psoriatic skin compared with non-lesional psoriatic skin ($n = 5$; Fig. 2c and S2b). To examine the phospho-STAT3 localization in lesional and non-lesional psoriatic skin, immunofluorescence imaging of the Tyr705- and Ser727-phosphorylated forms of STAT3 was carried out in biopsies from three different psoriatic patients. Both phosphorylated forms were detected in the nucleus of single keratinocytes in the whole epidermal layer. These phospho-STAT3-positive cells were not detected in non-lesional psoriatic skin (Fig. 2d).

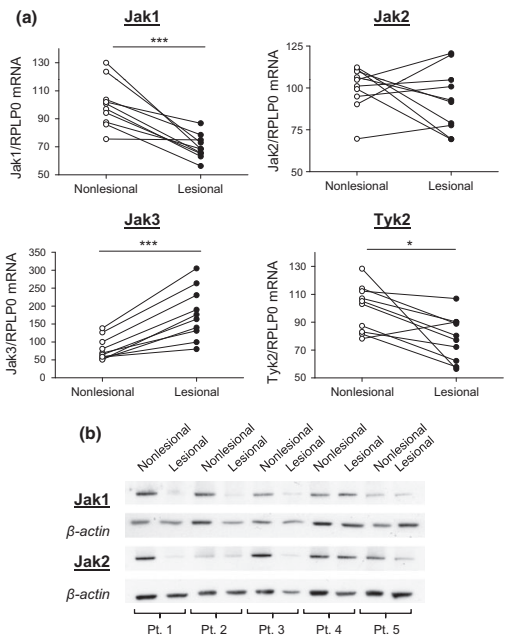

Figure 1. Jak and Tyk expression in plaque-type psoriasis. (a) mRNA expression, relative to housekeeping gene *RPLP0*, was analysed by RT-PCR. Bars indicate mean mRNA levels ± SEM in paired samples from non-lesional psoriatic skin (index 100) compared with lesional skin ($n = 10$). *$P < 0.05$; ***$P < 0.001$ versus non-lesional skin (b) Protein extracts from paired keratome biopsies from non-lesional and lesional psoriatic skin were analysed by Western blotting. The figure shows Jak1 and Jak2 blots from five psoriatic patients.

STAT3 phosphorylation modulates its transcriptional activity

To investigate whether increased STAT3 phosphorylation resulted in increased transcriptional activity, we firstly analysed the effect of different proinflammatory stimuli on STAT3 phosphorylation in normal human keratinocytes. After a time course study (Figure S3), IL-6 and IL-20 were identified to induce STAT3 Tyr705 phosphorylation in a time-dependent manner with a maximum effect after 30 min of stimulation. No effect on Ser727 was seen after IL-6 and IL-20 stimulation at any of the time points tested. In contrast, TNFα, TPA and UVB induced STAT3 Ser727 phosphorylation but had no effect on Tyr705 phosphorylation.

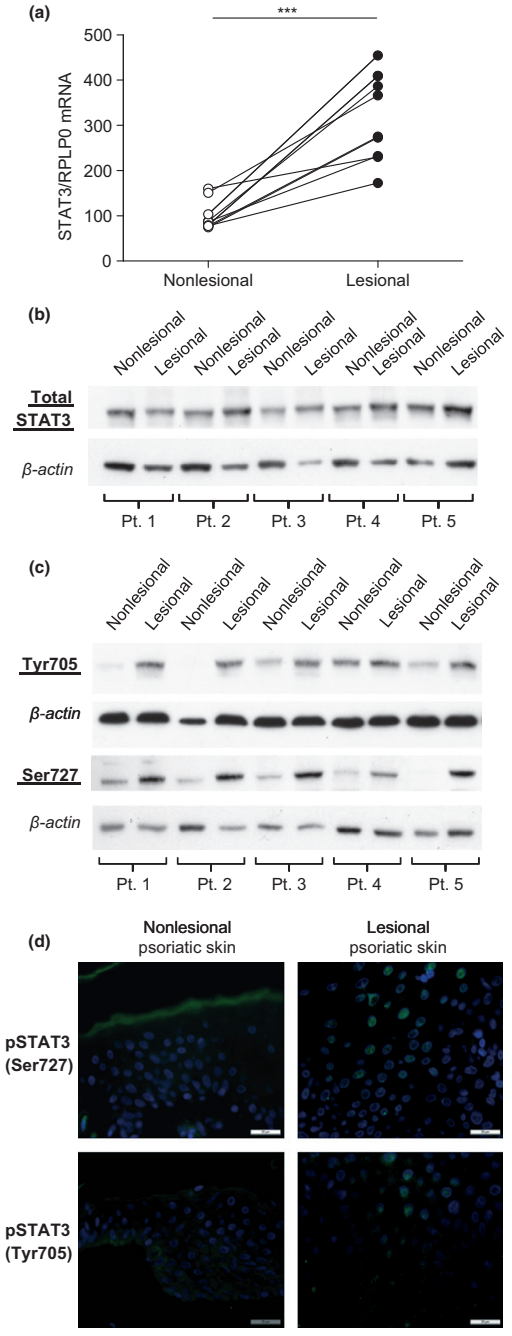

Figure 2. STAT3 expression and activation are increased in lesional psoriatic skin. (a) Total STAT3 mRNA relative to housekeeping gene *RPLP0*. Bars indicate mean mRNA level ± SEM in paired samples from non-lesional psoriatic skin (index 100) and lesional skin ($n = 10$). ***$P < 0.001$ *vs.* non-lesional skin (b) Western blots of total STAT3 protein from paired samples of non-lesional and lesional psoriatic skin from five patients. (c) Tyr705 and Ser727 phospho-STAT3 blots from five psoriatic patients. (d) Immunofluorescence staining of phospho-STAT3 (Tyr705 and Ser727) in non-lesional and lesional psoriatic skin. Green staining (Alexa Fluor 488) stains phospho-STAT3 (Tyr705 or Ser727), blue colour (40,6-diamidine-20-phenylindole dihydrochloride, DAPI) stains cell nuclei. Figures show representative fluorescence images from one of three psoriatic patients investigated. Scale bar = 100 μm.

Based on these findings, STAT3 transcriptional activity was studied after transfection of cultured normal human keratinocytes with the STAT3-promoter-luciferase-Renilla construct and subsequent stimulation with either IL-6 (50 ng/ml), IL-20 (10 nM) or TNFα (10 ng/ml) for different time periods. Three-h stimulation with IL-6 and IL-20 led to a significant 27- and 9-fold increase, respectively, in STAT3 driven transcriptional activity. This increase was maintained for at least 24 h. TNFα, however, could not induce any significant activation at any of the time points tested (Fig. 3a).

Given the different phosphorylation patterns exhibited by IL-6 and IL-20 in comparison with TNFα, we tested the combinations of IL-6 or IL-20 plus TNFα to assess whether the Ser727 phosphorylation of STAT3, induced by TNFα, could enhance the transcriptional activity produced by IL-6 and IL-20 and mediated by the Tyr705 phosphorylation. Surprisingly, TNFα exerted a modulating effect that differed depending on the stimuli it was combined with. It reduced IL-6 effects on STAT3 promoter activity by 25%, whereas it increased IL-20 stimulation by 10% (Fig. 3b).

Signalling pathways involved in STAT3 phosphorylation in normal human keratinocytes

To characterize the signalling pathways involved in STAT3 phosphorylation, normal human keratinocytes were preincubated with a Jak2 inhibitor (1,2,3,4,5,6-Hexabromocyclohexane), a p38 MAPK inhibitor (SB202190) or an ERK1/2 inhibitor (PD98059) for 45 min before stimulation with IL-6 (50 ng/ml), IL-20 (10 nM), TNFα (10 ng/ml) or TPA (100 nM) for 30 min. STAT3 phosphorylation was then analysed by Western blotting with primary antibodies detecting the Tyr705- or Ser727-phosphorylated forms.

In these conditions, the Jak2 inhibitor was able to completely block the STAT3 Tyr705 phosphorylation induced by IL-6 and IL-20. However, it did not have any effect on the Ser727 phosphorylation triggered by TNFα or TPA (Fig. 4a).

TNFα-induced phosphorylation of the Ser727 site was completely blocked by both the p38 MAPK and the ERK inhibitors, whereas the TPA-induced phosphorylation was only decreased by the ERK inhibitor. Interestingly, treatment with the p38 MAPK inhibitor enhanced TPA-mediated Ser727 phosphorylation of STAT3 (Fig. 4b and S4).

Discussion

Although different Jak inhibitors are being tested in clinical and preclinical trials for the treatment of psoriasis (6–8), the expression profile of Jak in lesional and non-lesional psoriatic skin has never been studied. Here, we report that all Jak proteins except Jak3 were downregulated in lesional psoriatic skin compared with non-lesional psoriatic skin. Jak3 is mainly expressed in lymphocytes (14), and therefore, its increase may be due to the increased infiltration of immunological cells in lesional psoriatic skin. However, interestingly

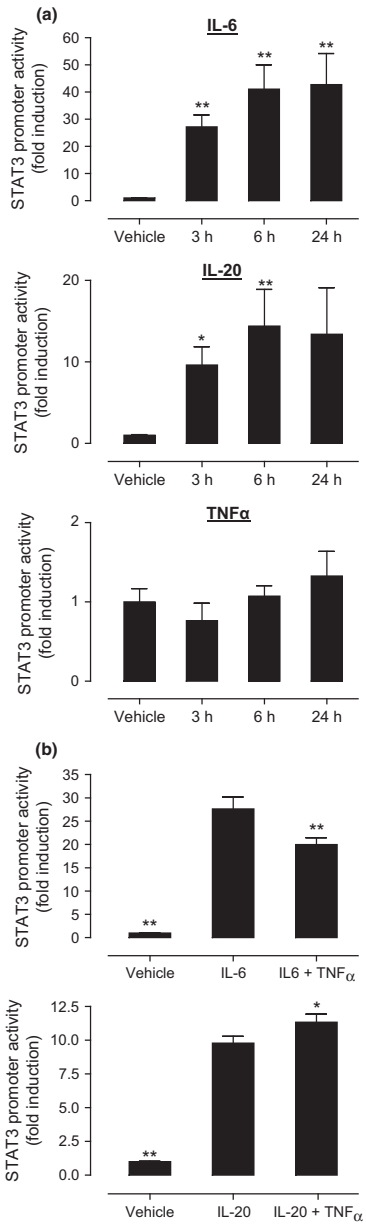

Figure 3. STAT3 transcriptional activity is increased by IL-6 and IL-20. Cultured normal human keratinocytes were transfected with STAT3-promoter-luciferase-Renilla plasmids. After transfection, cells were stimulated with either IL-6 (50 ng/ml), IL-20 (10 nM) or TNFα (10 ng/ml) for the indicated time points (a) or with a combination of them for 3 h (b). The activity was determined as a ratio between Firefly and Renilla luciferase activity. Results are shown as mean ± SEM. from at least three independent experiments. All measurements were performed in doublets. $*P < 0.05$; $**P < 0.01$ *vs.* vehicle-treated cells.

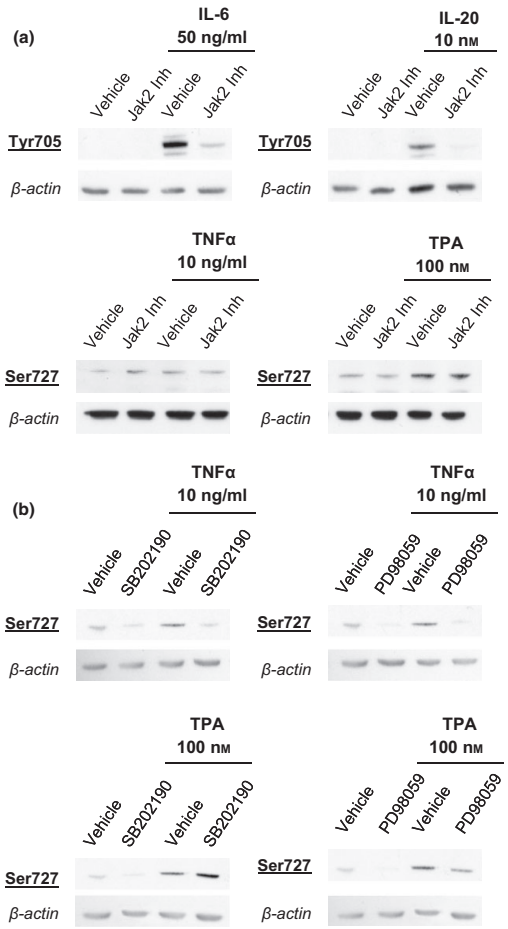

Figure 4. Different signalling pathways are involved in STAT3 phosphorylation in normal human keratinocytes. Cells were preincubated with a Jak2 inhibitor (1,2,3,4,5,6-Hexabromocyclohexane, 50 μM) (a), a p38 MAPK inhibitor (SB202190, 10 μM) or an ERK1/2 inhibitor (PD98059, 50 μM) (b) for 45 min and stimulated with IL-6 (50 ng/ml), IL-20 (10 nM), TNFα (10 ng/ml) or TPA (100 nM) for 30 min. STAT3 phosphorylation was then analysed by Western blotting. One representative blot out of three independent experiments is shown.

Tofacitinib (CP-690,550), that is currently being tested in clinical phase III studies for psoriasis, is a Jak1/3 inhibitor (8).

Several studies have implicated STAT3 in regulating fundamental cellular processes such as proliferation, differentiation, survival and apoptosis (15). An altered regulation of these processes in keratinocytes is one of the key features in psoriasis. In accordance with previous studies (10,11), STAT3 expression was shown to be upregulated in psoriatic lesions. Moreover, we have proven an increase in not only the Tyr705 but also the Ser727 phosphorylation of STAT3 throughout all the epidermal layers in lesional psoriatic skin compared with non-lesional psoriatic skin. These findings strongly suggest an increased STAT3 activity within the

keratinocyte compartment of psoriatic lesions. Ser727-phosphorylated STAT3 has been suggested to contribute to oxidative phosphorylation in the mitochondria, and augments cell transformation by oncogenic Ras (21) – an effect that is independent of STAT3 Tyr705 phosphorylation.

In addition, the phosphorylation pattern exhibited by main STAT3 activators was investigated in normal human keratinocytes and furthermore, the implications in terms of transcriptional activity for each of the phosphorylation sites were studied. Tyr705 phosphorylation driven by IL-20 and IL-6 was shown to have a great impact on STAT3 activity, whereas Ser727 phosphorylation induced by TNFα did not have any effect. However, Ser727 phosphorylation may play an important role in modulating IL-6 and IL-20 responses as seen in the luciferase assays. Although tyrosine phosphorylation of STAT3 is accompanied by serine phosphorylation in a variety of cells, the functional significance of Ser727 phosphorylation remains controversial. Some studies suggest that it enhances STAT3 transcriptional activity, whereas other reports demonstrate that it has an inhibitory effect (22–24). In agreement with our results, the requirement for a phosphorylated Ser727 may vary with the promoter and/or cellular context (Reviewed in 16). Furthermore, these differences could also be independent of Ser727 phosphorylation and instead be directly mediated by the activation of the MAPK pathways involved in the phosphorylation of this site. MAPKs may have a negative effect on upstream tyrosine kinases such as EGF receptor, Src and Janus kinases or a positive effect on suppressors of cytokine signalling (SOCS) (25,26). Further experiments will need to be conducted to fully elucidate the physiological and pathophysiological role of Ser727 phosphorylation in STAT3 function in human keratinocytes.

Finally, we investigated the kinases involved in STAT3 phosphorylation in normal human keratinocytes. The tyrosine phosphorylation of STAT3 was mediated by Jak2 after both IL-6 and IL-20 challenge, demonstrating a pivotal role of this kinase in STAT3 activation and making it an appealing therapeutic target for the treatment of psoriasis. No effect of this kinase on Ser727 phosphorylation was observed. Serine phosphorylation of one STAT may occur through different signal transduction pathways depending on the stimulus; even more, it may involve the integration of multiple kinase pathways (25). ERKs, particularly ERK2, have been proven to be STAT3 targeting kinases or upstream of a STAT3 Ser727 targeting kinase in other cell lines (16). Our findings support an important role of these kinases in STAT3 Ser727 phosphorylation in normal human keratinocytes because none of the tested stimuli lead to phosphorylation of this site when cells were pretreated with the ERK1/2 inhibitor, PD98059, before stimulation. However, pretreatment with the p38 MAPK inhibitor SB202190 only abolished TNFα-induced STAT3 Ser727 phosphorylation, suggesting that TNFα induction is dependent on ERK and p38 MAPK signalling pathways, whereas TPA-induced phosphorylation is not dependent on p38 MAPK activity.

These data clearly demonstrate the complexity of how different proinflammatory mediators, for example, cytokines use intracellular signalling pathways to communicate information to the nucleus where different signalling pathways converge and integrate their information through regulation of transcriptional activity of one or more specific transcription factors like, for example STAT3.

Taken together, this study has shed light on Jak and STAT3 expression and activation in psoriasis and the underlying mechanism involved. A modulating effect in STAT3 activity has been demonstrated for the Ser727 phosphorylation in normal human keratinocytes, suggesting a regulatory effect of the MAPK family in STAT3 activation. The results also demonstrate the complexity of signal transduction, with many different cytokines and stimuli activating different signalling pathways that subsequently integrate and determine the net transcriptional activity of a specific transcription factor.

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Author contributions

Rosa María Andrés, Claus Johansen, Knud Kragballe and Lars Iversen designed the research study. Rosa María Andrés and Anne Hald performed the research and analysed the data. Rosa María Andrés wrote the article.

Conflict of interests

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Jak and Tyk2 protein expression.

Figure S2. STAT3 expression and activation in psoriatic skin.

Figure S3. Time course study of STAT3 phosphorylation.

Figure S4. Densitometric analysis of the Western blotting band intensity from the inhibitor study of STAT3 phosphorylation.

Artículo 2

STAT1 expression and activation is increased in lesional psoriatic skin

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STAT1 expression and activation is increased in lesional psoriatic skin

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Conflicts of interest
None declared.

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Summary

Background The JAK (Janus kinase)/STAT (signal transducer and activator of transcription) signalling pathway is known to play an important role in many cellular processes including inflammation. The activation of STAT1 is dependent on tyrosine 701 and serine 727 phosphorylation, which leads to the formation of the STAT dimer and modulation of STAT1 activity, respectively.

Objective To determine STAT1 expression and activation in psoriatic skin.

Methods Biopsies were collected from patients with psoriasis. mRNA expression was evaluated by quantitative polymerase chain reaction, whereas the protein and phosphorylation level of STAT1 were evaluated by Western blotting. STAT1 localization was determined by immunofluorescence analysis and STAT1-induced transcriptional activity was analysed in cultured human keratinocytes using a reporter assay.

Results The expression of STAT1 was demonstrated to be significantly increased at both mRNA and protein level in lesional psoriatic skin. In addition, the phosphorylation level of STAT1(Tyr701) and STAT1(Ser727) was significantly increased in lesional compared with nonlesional psoriatic skin. Luciferase assays showed a significant induction of the STAT1-induced transcriptional activity when cultured human keratinocytes were stimulated with either interferon (IFN)-α or IFN-γ. STAT1(Ser727) phosphorylation induced by IFN-α, IFN-γ or ultraviolet B was mediated by a protein kinase C (PKC)-δ- and p38 mitogen-activated protein kinase-dependent mechanism in human keratinocytes, whereas IFN-α-induced STAT1(-Tyr701) phosphorylation was mediated by a PKC-δ-dependent mechanism.

Conclusions This study demonstrates for the first time that the phosphorylation level of STAT1(Tyr701) and STAT1(Ser727) is increased in lesional psoriatic skin. In addition, specific signalling pathways leading to this phosphorylation have been identified. Together, our data indicate an important role of STAT1 in the pathogenesis of psoriasis.

Psoriasis is a chronic, inflammatory immune-mediated skin disease with a prevalence of 2–3%. The skin disease is characterized by skin-infiltrating lymphocytes, hyperproliferation and an abnormal differentiation of the keratinocytes.[1–3] Several studies have shown that alterations in specific signal transduction pathways may be part of the explanation for the inflammation seen in psoriasis.[4–6] Abnormalities in the expression/activity of different transcription factors involved in the JAK (Janus kinase)/STAT (signal transducer and activator of transcription) signalling pathway have been hypothesized to play a role in the pathogenesis of psoriasis.[7–9] In support of this, topical treatment with a JAK1/JAK2 inhibitor has recently been evaluated on animal models in a preclinical trial and identified JAK1 and JAK2 as novel targets in the treatment of psoriasis.[10] In addition, oral treatment of CD18 mutant PL/J mice with a JAK3 inhibitor showed a dose-dependent attenuation in the induced skin inflammation.[11]

STAT1 is involved in type I and type II interferon (IFN) signalling and belongs to the STAT family, which consists of seven different but homologous members: STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6.[12] Activation of the STAT1 signalling pathway is initiated upon cytokine binding to the extracellular part of its cognate receptor. This leads to a dimerization of the receptor and autophosphorylation of the associated JAK proteins.[13] Type II IFNs activate the IFN-γ receptor complex, leading to a tyrosine 701 phosphorylation of STAT1 through a JAK1/JAK2-dependent mechanism and subsequently formation of a STAT1 homodimer.[14] In contrast, type I IFNs activate JAK1 and tyrosine kinase-2 phosphorylation, which in turn activates and phosphorylates STAT1 and

STAT2 on a tyrosine 701 site leading to dimerization of the STAT1/STAT2 heterodimer, and subsequently formation of IFN-stimulated gene factor 3 (ISGF3).[15] The STAT1 homodimer binds to promoter regions, which have IFN-γ-activated sites (GAS), whereas the ISGF3 complex binds to IFN-stimulated response elements (ISREs) at the promoter regions of specific target genes. Transcriptional activity of STAT1 is believed to be modulated through phosphorylation of the serine 727 residue.[16] The functional role of the serine phosphorylation is, however, still unknown but it does not seem to influence the DNA-binding affinity or the nuclear translocation of the STAT dimer.[17,18]

In this study, we demonstrate that both the expression and the activity of STAT1 are significantly increased in lesional psoriatic skin compared with nonlesional psoriatic skin. We also demonstrate that STAT1 phosphorylation is induced through a p38 mitogen-activated protein kinase (MAPK)- and protein kinase C (PKC)-δ-dependent mechanism in cultured human keratinocytes. Moreover, luciferase assays showed significant induction of STAT1 transcriptional activity when cultured human keratinocytes were stimulated with IFN-α or IFN-γ.

Materials and methods

Biopsies

For protein analysis, keratome biopsies from five different patients with psoriasis were obtained as previously described[5] using the Zimmer® electronic dermatome (Zimmer Orthopaedic, Dover, OH, U.S.A.). The dermatome was adjusted according to the clinically evaluated thickness of the individual plaques in order to obtain the full epidermis and only minor parts of the dermis in each biopsy and when obtaining nonlesional biopsies the depth of cut was reduced. For quantitative reverse transcription–polymerase chain reaction (qPCR), 4-mm punch biopsies from nine patients with psoriasis were taken from the centre of a chronic plaque and nonlesional psoriatic skin and immediately snap-frozen in liquid nitrogen and stored until further use. The five patients used for protein analysis were different from the nine patients used for the mRNA analysis.

This study was conducted according to the Declaration of Helsinki principles. The local ethical committee of Aarhus approved all described studies, and signed informed consent was obtained from each patient.

Cell cultures

Human keratinocytes were obtained by trypsinization of skin samples from patients undergoing plastic surgery. Second-passage keratinocytes were grown in an incubator at 37 °C and 5% CO_2 in keratinocyte serum-free medium (K-SFM; Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) with added gentamicin and supplements until 60–80% confluent. Twenty-four hours before stimulation the medium was changed to keratinocyte basal medium (the same as K-SFM, but without growth factors) in which the cells were stimulated. Cells were stimulated with either IFN-α (1000 U mL^{-1}), IFN-γ (50 ng mL^{-1}), interleukin (IL)-6 (50 ng mL^{-1}) or ultraviolet (UV) B (250 J m^{-2}). Vehicle [phosphate-buffered saline (PBS) with 0·15% bovine serum albumin] was added to the control. The UV source was a linear bank of UVB fluorescence tubes emitting UVB light (Phillips TL12; Phillips, Eindhoven, the Netherlands). In some experiments the keratinocytes were pretreated for 45 min with the p38 MAPK inhibitor, SB202190 (10 μmol L^{-1}), or the PKC-δ inhibitor, rottlerin (6 μmol L^{-1}; both purchased from Calbiochem, San Diego, CA, U.S.A.) before stimulation. In each experimental setting, different donors were used for the different keratinocyte cultures included.

Whole cell lysates

The keratinocytes were washed with ice-cold PBS before harvest in 1× cell lysis buffer [20 mmol L^{-1} Tris-base (pH 7·5), 150 mmol L^{-1} NaCl, 1 mmol L^{-1} ethylenediamine tetraacetic acid (EDTA), 1 mmol L^{-1} ethylene glycol bis-(beta-aminoethyl ether) N,N,N′,N′-tetraacetic acid, 1% Triton X-100, 2·5 mmol L^{-1} sodium pyrophosphate, 1 mmol L^{-1} β-glycerol phosphate, 1 mmol L^{-1} Na_3VO_4, 1 μg mL^{-1} leupeptin] containing 1 mmol L^{-1} phenylmethylsulphonyl and Complete®-EDTA-free protease inhibitor (Roche Diagnostics, Mannheim, Germany). Cell lysates were incubated on ice for 5 min, then sonicated and centrifuged at 10 000 *g* for 10 min after which the supernatants containing the proteins were removed into new Eppendorf tubes (Eppendorf, Hamburg, Germany).

Western blotting

The protein concentration was determined by a Bradford assay. Equal protein amounts were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then blocked and incubated with the primary antibodies [STAT1, p-STAT1(Tyr701) and p-STAT1(Ser727), cat. nos 9172, 9171 and 9177, respectively; Cell Signaling Technology, Beverly, MA, U.S.A.] overnight at 4 °C. Primary antibodies were detected using antirabbit IgG horseradish peroxidase-linked antibody (cat. no. 7074; Cell Signaling Technology) and ECL reaction (Amersham Biosciences, Chalfont St Giles, U.K.) according to the manufacturers' instructions. Densitometric analysis of the band intensity was carried out using Kodak 1D Analysis Software (Kodak, Rochester, NY, U.S.A.).

RNA isolation

Punch biopsies were transferred to 1 mL of −80 °C cold RNAlater®-ICE (Applied Biosystems, Foster City, CA, U.S.A.). Twenty-four hours before RNA purification samples were transferred to −20 °C. Upon RNA purification biopsies were removed from RNAlater-ICE and transferred to SV RNA lysis buffer supplemented with β-mercaptoethanol (SV Total RNA Isolation System; Promega, Madison, WI, U.S.A.) and homogenized. RNA purification including DNase treatment of the

samples was completed according to the manufacturer's instructions (SV Total RNA Isolation System; Promega). The RNA was stored at −80 °C until further use.

Quantitative polymerase chain reaction

The qPCR was performed as previously described.[19] When formatting the cDNA by reverse transcription we used Taqman® Reverse Transcription Reagents (Applied Biosystems). *STAT1* and *AIM2* (absent in melanoma 2) mRNA expression were analysed using TaqMan® Gene Expression Assays Hs01013996_m1 and Hs00915710_m1, respectively (Applied Biosystems). The probe was a FAM-labelled MGB probe with a nonfluorescent quencher. The expression level of the housekeeping gene ribosomal protein large P0 (*RPLP0*) was determined using the TaqMan® Gene Expression Assays (Hs99999902_m1) (Applied Biosystems).

The PCR master mix consisted of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen Life Technologies). Each gene was analysed in triplicate. The real-time PCR machine was a Rotorgene-3000 (Corbett Research, Sydney, NSW, Australia). Reactions were run as singleplex. Relative gene expression levels were determined by using the relative standard curve as outlined in User Bulletin no. 2 (ABI Prism 7700 sequencing detection system; Applied Biosystems). The standard curve, from which the relative amounts of target mRNA was calculated was made of fourfold serial dilutions of total RNA from a punch biopsy from a psoriatic plaque.

Immunofluorescence analysis

Paraffin-embedded tissue samples (4-μm sections) from lesional and nonlesional psoriatic skin were used. Immunofluorescence analysis was conducted as previously described.[19] Briefly, the tissue samples were deparaffinized and heated at 95 °C for 10 min in 10 mmol L^{-1} sodium citrate buffer (pH 6·0) for antigen retrieval. The samples were then blocked for 30 min with Image-iT™ FX Signal Enhancer (Invitrogen Life Technologies). The primary antibodies used were p-STAT1(Tyr701) and p-STAT1(Ser727), cat. nos 9167 and 9177, respectively (Cell Signaling Technology). The secondary antibody was Alexa Fluor 488 goat antirabbit (Invitrogen Life Technologies). Samples were evaluated by epifluorescence microscopy (Leica, Heidelberg, Germany). As negative controls, sections were incubated with blocking buffer without the primary antibody or with normal rabbit IgG.

Transfection

Keratinocytes were grown in a 24-well plate for 24 h to a confluence of about 60%. Before transfection the medium was changed to basal medium without gentamicin. pCIS-CK (Agilent Technologies, Inc., Santa Clara, CA, U.S.A.) served as a negative control; the plasmid contains a luciferase reporter gene but does not contain any *cis*-acting DNA elements. Keratinocytes were transfected using two different plasmids, the pISRE-Luc plasmid or the p-GAS-Luc plasmid both purchased from Agilent Technologies, and the internal control *Renilla* luciferase expression plasmid (pRL-TK; Promega) using Fugene 6 transfection reagent (Roche Diagnostics) according to the manufacturer's protocol. Twenty-four hours after transfection the samples were stimulated using IFN-α, IFN-γ, UVB or IL-6.

Dual luciferase reporter assay

Cell medium was removed from the wells and 100 μL diluted Passive Lysis Buffer (Promega) was added. Cells were then incubated under gentle agitation for 15 min. Then 20 μL from each sample was added onto a 96-well plate and the luciferase activity was measured using the dual luciferase assay system (Promega) on a Fluoroscan Ascent FL (Bie & Berntsen, Rødovre, Denmark) according to the manufacturer's protocol.

Statistical analysis

For statistical analysis a Student's *t*-test was performed. To test for normal distribution a probability test was made. A value of $P < 0{\cdot}05$ was regarded as statistically significant.

Results

STAT1 expression is elevated in lesional psoriatic skin

To characterize *STAT1* mRNA expression in psoriatic skin, RNA from punch biopsies obtained from lesional and nonlesional psoriatic skin was isolated and analysed by qPCR. In Figure 1a, qPCR results from nine different patients with psoriasis are depicted, all showing an increased mRNA expression of *STAT1* in lesional psoriatic skin compared with nonlesional psoriatic skin. The mean *STAT1* mRNA expression was found to be significantly increased (approximately fivefold) in lesional compared with nonlesional psoriatic skin ($P = 0{\cdot}002$) (Fig. 1b).

To investigate whether or not the increased mRNA expression of *STAT1* was paralleled by an increased accumulation of STAT1 protein, Western blotting analysis of protein extracts isolated from paired keratome biopsies taken from patients with psoriasis was performed (Fig. 1c). The protein level of STAT1 was found to be significantly augmented in lesional compared with nonlesional psoriatic skin ($P = 0{\cdot}005$) showing a mean increase of approximately 3·3-fold (Fig. 1d).

Phosphorylated (p)-STAT1(Tyr701) and p-STAT1(Ser727) are increased in lesional psoriatic skin

Two different phosphorylation sites on STAT1 are known: tyrosine 701 and serine 727. To study the activity of STAT1 in psoriatic skin we isolated whole-cell extracts from lesional and nonlesional psoriatic keratome biopsies.

By Western blotting analysis, using antibodies recognizing phosphorylated/activated STAT1 [p-STAT1(Tyr701) and p-STAT1(Ser727)], we demonstrated that the level of p-STAT1(Tyr701) was significantly increased in lesional psoriatic skin compared with nonlesional psoriatic skin ($P < 0{\cdot}05$).

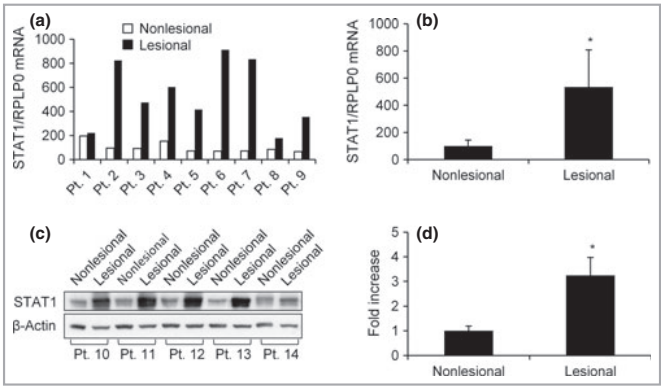

Fig 1. STAT1 expression in psoriatic skin. (a, b) mRNA expression of *STAT1* was analysed in paired biopsies obtained from lesional and nonlesional psoriatic skin by quantitative polymerase chain reaction. The mRNA expression of the housekeeping gene *RPLP0* was used for normalization. (c, d) Western blot analysis was performed on whole-cell extracts from paired lesional and nonlesional psoriatic keratome biopsies. To secure equal loading the membrane was stripped and probed with β-actin. Densitometric analysis of the band intensity was carried out and values were normalized to β-actin. *P < 0·01 compared with nonlesional skin. Pt, patient.

Results from five different patients with psoriasis are depicted in Figure 2a, showing an almost sevenfold increase in the level of p-STAT1(Tyr701) protein content in lesional psoriatic skin compared with nonlesional psoriatic skin (Fig. 2b).

Also the protein level of p-STAT1(Ser727) was demonstrated to be significantly increased (P = 0·005) in lesional psoriatic skin, showing a mean increase of approximately 26-fold (Fig. 2c, d).

Immunofluorescence staining of activated/phosphorylated STAT1 in psoriatic skin

Due to the expression profile of STAT1 mRNA and protein in psoriatic skin we next wanted to examine the localization of the two forms of activated/phosphorylated STAT1, p-STAT1 (Tyr701) and p-STAT1(Ser727), in psoriatic skin. For that purpose, we used 4-μm sections of paraffin-embedded punch biopsies from lesional and nonlesional psoriatic skin.

Immunofluorescence analysis of p-STAT1(Tyr701) showed single positively stained cells localized only in the epidermis of lesional psoriatic skin, whereas no nuclear staining was observed in nonlesional psoriatic skin. p-STAT1(Ser727) showed nuclear staining scattered throughout the entire layers of the epidermis, but not the dermis in lesional psoriatic skin compared with less positively stained cells in the epidermis of nonlesional psoriatic skin (Fig. 3). No epidermal cells stained positive when using the IgG control.

Interferon (IFN)-α, IFN-γ, ultraviolet B and interleukin 6 stimulation leads to increased activation/phosphorylation of STAT1 in cultured human keratinocytes

To further characterize STAT1 we next used cultured normal human keratinocytes stimulated with IFN-α, IFN-γ, UVB or IL-6 for different time points.

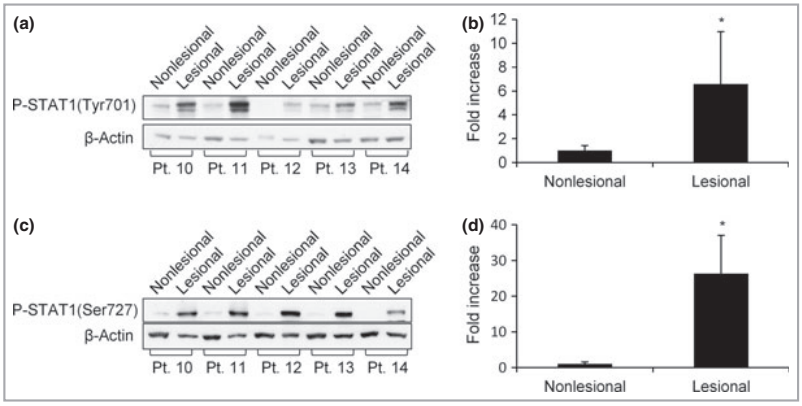

Fig 2. STAT1 phosphorylation in psoriatic skin. Whole-cell protein extracts were prepared from paired psoriatic keratome biopsies and analysed by Western blotting. The protein extracts were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis. After blotting the membranes were probed with an antibody recognizing (a) p-STAT1(Tyr701) or (c) p-STAT1(Ser727). (b) p-STAT1(Tyr701) in lesional psoriatic skin compared with nonlesional skin (n = 5, *P < 0·05). (d) p-STAT1(Ser727) in lesional psoriatic skin compared with nonlesional psoriatic skin (n = 5; *P = 0·005). Equal loading was assessed by detecting the protein level of β-actin. Pt, patient.

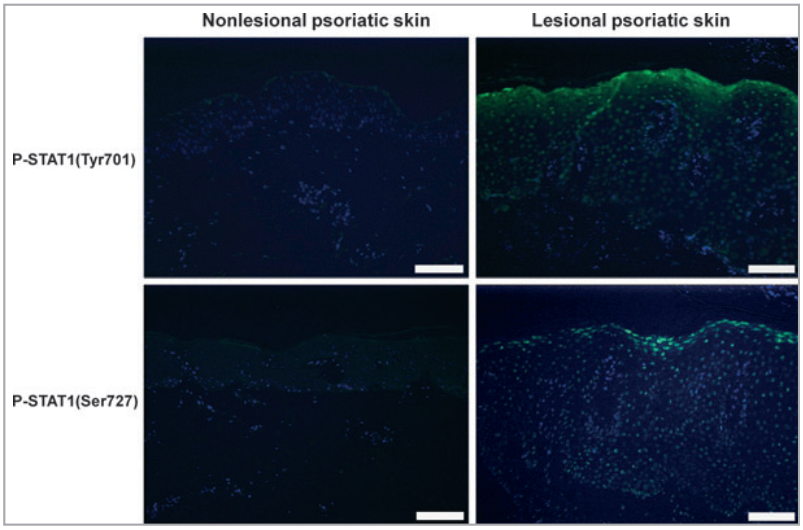

Fig 3. Localization of activated/phosphorylated STAT1 in psoriatic skin. Immunofluorescence analysis was performed on paraffin-embedded punch biopsies from nonlesional and lesional psoriatic skin to localize the activated forms of STAT1: p-STAT1(Tyr701) and p-STAT1(Ser727). Nuclear staining was performed using 4′,6-diamidine-2′-phenylindole dihydrochloride. A green colour (Alexa Fluor 488) represents the activated/phosphorylated forms of STAT1. Three sets of paired biopsies from three different patients were investigated. Scale bar = 100 μm.

IFN-α was demonstrated to induce phosphorylation of both STAT1(Tyr701) and STAT1(Ser727) in a time-dependent manner with a maximum increase seen after 1 h of stimulation. Six hours after stimulation with IFN-α the phosphorylation level of STAT1(Tyr701) and STAT1(Ser727) had almost returned to baseline (Fig. 4a). IFN-γ also induced a rapid and time-dependent phosphorylation of STAT1(Tyr701) and STAT1(Ser727). The maximum increase of p-STAT1(Tyr701) and p-STAT1(Ser727) was observed after 15 min and 1 h, respectively (Fig. 4b).

UVB stimulation of cultured human keratinocytes demonstrated a clear induction in the phosphorylation level of STAT1(Ser727) with a maximum increase after 1 h. In contrast, UVB stimulation of the keratinocytes did not lead to any phosphorylation of STAT1(Tyr701) (Fig. 4c).

IL-6 is known to be a strong inducer of STAT3(Tyr705) phosphorylation.[20,21] Here we investigated if IL-6 stimulation of cultured human keratinocytes had any effect on the phosphorylation level of STAT1. Interestingly, IL-6 was demonstrated to increase the phosphorylation of STAT1 (Tyr701) in a time-dependent manner, with a maximum increase after 30 min of stimulation. In contrast, IL-6 had no effect on the serine 727 phosphorylation site of STAT1 (Fig. 4d).

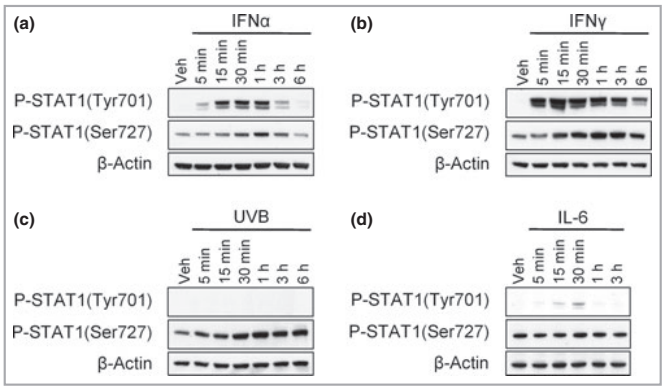

Fig 4. The effect of interferon (IFN)-α, IFN-γ, ultraviolet (UV) B and interleukin (IL)-6 on the phosphorylation level of STAT1. Cultured human keratinocytes were stimulated with either (a) IFN-α, (b) IFN-γ, (c) UVB or (d) IL-6 for the indicated time periods. Protein extracts were isolated, separated on a gradient gel by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and targeted using an antibody recognizing either p-STAT1(Tyr701) or p-STAT1(Ser727). Equal loading was confirmed by incubation with β-actin. Veh, vehicle.

Interferon (IFN)-α and IFN-γ induce transcriptional activity in cultured human keratinocytes

To test whether the induced phosphorylation of STAT1 correlated with increased STAT1 transcriptional activity, we conducted a luciferase assay.

Keratinocytes transfected with the pISRE-Luc plasmid and subsequently stimulated with IFN-α showed a modest but significant ($P = 0{\cdot}01$) twofold increase in the luciferase activity after 3 and 6 h (Fig. 5a). Interestingly, when keratinocytes were transfected with the pGAS-Luc plasmid and subsequently stimulated with IFN-γ for 3 and 6 h we found a significant increase in the luciferase activity of approximately 21- and 29-fold, respectively ($P = 0{\cdot}01$) (Fig. 5b). Neither UVB nor IL-6 stimulation correlated with increased activity of the reporter construct using either the pISRE-Luc plasmid or the pGAS-Luc plasmid (Fig. 5c–f).

STAT1 phosphorylation is induced through a p38 mitogen-activated protein kinase- and a protein kinase-δ-dependent mechanism

p38 MAPK and PKC-**δ** have previously been demonstrated to be involved in STAT1 phosphorylation in different cell types, but their role in STAT1 phosphorylation has not been explored in keratinocytes.[18] To investigate the role of these two kinases in the phosphorylation of STAT1 in the skin we pretreated cultured human keratinocytes for 45 min with a p38 MAPK inhibitor (SB202190) or a PKC-**δ** inhibitor (rottlerin) before stimulation with IFN-α, IFN-γ or UVB for 1 h.

Preincubation of keratinocytes with SB202190 before stimulation with IFN-α resulted in a minor but significant inhibition in the phosphorylation level of STAT1(Ser727) ($P = 0{\cdot}03$) but not the phosphorylation level of STAT1 (Tyr701) (Fig. 6a and Fig. S1). In contrast, preincubation with rottlerin significantly inhibited both the STAT1(Tyr701) and STAT1(Ser727) phosphorylation (Fig. 6b and Fig. S1).

Next, we analysed the effect of the two inhibitors on the IFN-γ-induced phosphorylation of STAT1. We demonstrated that preincubation of keratinocytes with SB202190 or rottlerin led to a small but significant ($P = 0{\cdot}04$ and $P = 0{\cdot}003$, respectively) decrease in the phosphorylation of the serine 727 site, but not on the tyrosine 701 site (Fig. 6c, d and Fig. S1).

The UVB-induced phosphorylation of STAT1(Ser 727) was found to be mediated by a signalling pathway involving both p38 MAPK and PKC-**δ**, because SB202190 and rottlerin significantly ($P = 0{\cdot}02$ and $P = 0{\cdot}04$, respectively) reduced the UVB-induced phosphorylation (Fig. 6e, f and Fig. S1).

To analyse if the inhibition of STAT1 phosphorylation also led to an inhibition of transcriptional activity, we next analysed the involvement of the p38 MAPK and PKC-**δ** signalling pathway on *AIM2*. Stimulation of keratinocytes with IFN-α or IFN-γ resulted in a significant ($P = 0{\cdot}001$ and $P = 0{\cdot}004$, respectively) increase in the mRNA expression of *AIM2* (Fig. 6g, h). Preincubation with rottlerin lead to a significant ($P = 0{\cdot}002$) reduction in the IFN-α-induced *AIM2* mRNA expression. Inhibition of the p38 MAPK pathway also resulted in a significant ($P = 0{\cdot}03$) inhibition of *AIM2* mRNA expression although the reduction was less pronounced compared with the reduction seen with rottlerin (Fig. 6g), supporting the phosphorylation data of STAT1 as seen in Figure 6a and b. Also the IFN-γ-induced *AIM2* mRNA expression was significantly inhibited by both rottlerin and SB202190 ($P = 0{\cdot}02$ and $P = 0{\cdot}03$, respectively) (Fig. 6h). The inhibition of IFN-γ-

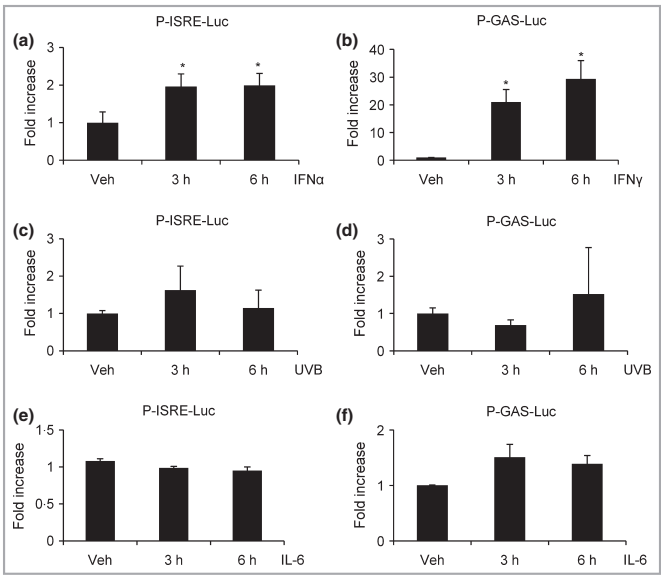

Fig 5. Induction of transcriptional activity in keratinocytes. Transcription activity was examine by dual luciferase assays, which were carried out using cultured human keratinocytes transfected with either the pISRE-Luc plasmid or the pGAS-Luc plasmid and subsequently stimulated with (a) interferon (IFN)-α or (b) IFN-γ, respectively. The effects of ultraviolet (UV) B and interleukin (IL)-6 stimulation were investigated using pISRE-Luc (c, e) and pGAS-Luc (d, f) separately. Twenty-four hours after transfection, cells were stimulated for 3 and 6 h with UVB or IL-6. Each study was normalized to *Renilla* and carried out in duplicates on six different keratinocyte cultures. The fold increase is presented as mean ± SD; $n = 3–6$; *$P = 0{\cdot}01$. ISRE, interferon-stimulated response elements; GAS, interferon-γ-activated sites.

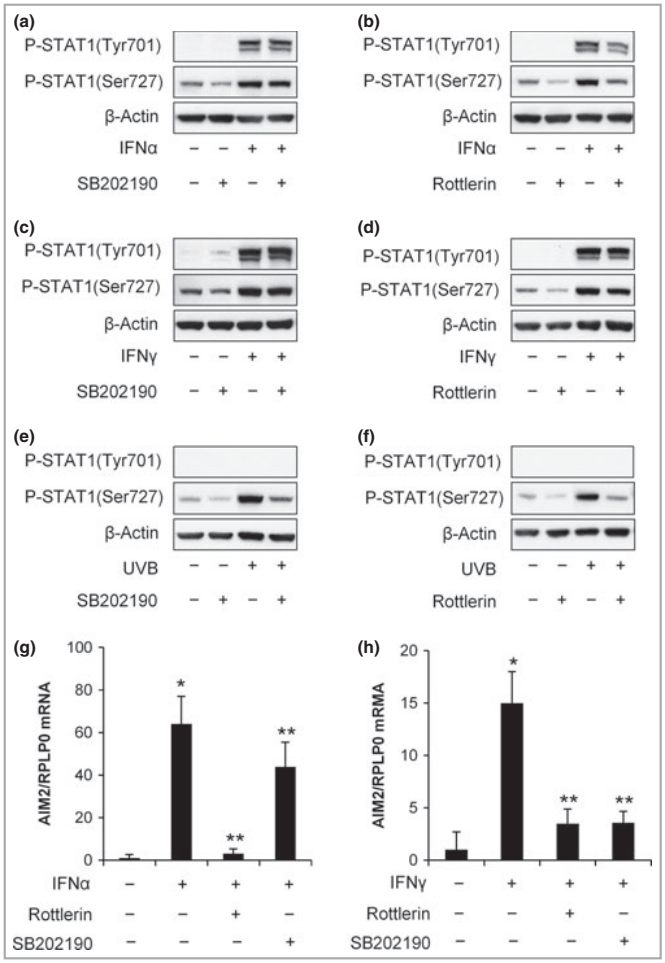

Fig 6. Effect of the kinase inhibitors SB202190 and rottlerin on the level of phosphorylated STAT1 and AIM2 mRNA expression. Whole-cell extracts from cultured human keratinocytes were used to study the effect of SB202190 and rottlerin on the activation of STAT1. Forty-five minutes prior to stimulation with either interferon (IFN)-α (a, b), IFN-γ (c, d) or ultraviolet (UV) B (e, f), human keratinocytes were incubated with the SB202190 or rottlerin. The protein extracts were then isolated, separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and subsequently targeted with antibodies recognizing either p-STAT1(Tyr701) or p-STAT1(Ser727) ($n = 3–5$). (g, h) Keratinocytes were treated with or without SB202190 or rottlerin before being stimulated with (g) IFN-α or (h) IFN-γ for 6 h. RNA was isolated and the mRNA expression of *AIM2* analysed by quantitative polymerase chain reaction. The mRNA expression of RPLP0 was used for normalization. Results represent the mean ± SD from experiments conducted on three different keratinocyte cultures. *$P < 0{\cdot}05$ compared with vehicle-treated keratinocytes; **$P < 0{\cdot}05$ compared with IFN-α- or IFN-γ-treated keratinocytes.

induced *AIM2* mRNA expression mediated by rottlerin and SB202190 was almost the same, fitting nicely with the STAT1 phosphorylation data, showing only a small inhibitory effect of SB202190 and rottlerin on the phosphorylation of STAT1(-Ser727) but not on STAT1(Tyr701) as seen in Figure 6c and d.

Discussion

The JAK/STAT signalling pathway is an intracellular signalling pathway, known to be involved in inflammatory processes.[22] Recently, studies have identified altered activation of specific intracellular signalling pathways resulting in the inflammation seen in psoriasis.[6]

In this study we present two essential and novel findings, which add new knowledge to the role of the JAK/STAT signalling pathway in the pathogenesis of psoriasis. Firstly, we demonstrate that there is an increased level of the phosphorylated forms of STAT1(Tyr701) and STAT1(Ser727) in lesional compared with nonlesional psoriatic skin. Secondly, the two kinases p38 MAPK and PKC-**δ** were demonstrated to be strongly involved in the phosphorylation of STAT1 in cultured human keratinocytes.

In agreement with our results, previous studies have shown increased levels of STAT1 in lesional psoriatic skin as determined by immunohistochemistry and gene array analysis.[7,23] In this study we have characterized STAT1 even further, and shown not only an increased mRNA and protein expression of STAT1 in lesional psoriatic skin, but also an elevated level of the two phosphorylated forms of STAT1, STAT1(Tyr701) and STAT1(Ser727). These data demonstrate that STAT1 is not only altered at a transcriptional level in psoriatic skin, but also at a post-translational level, strongly indicating that STAT1 plays an important role in the pathogenesis of psoriasis.

p38 MAPK and PKC-**δ** have previously been shown to be involved in serine 727 phosphorylation of STAT1.[24] Moreover, p38 MAPK is activated in psoriasis as previously demonstrated[25] and PKC-**δ** has been hypothesized to be an upstream regulator of p38 MAPK in U-266, Molt-4 and endothelial cells.[18,26] Therefore, cultured human keratinocytes were pretreated with a p38 MAPK inhibitor (SB202190) or a PKC-**δ** inhibitor (rottlerin) and subsequently stimulated with IFN-α, IFN-γ or UVB, in order to characterize the role of these two kinases in the activation of STAT1. We demonstrated that both p38 MAPK and PKC-**δ** are involved in the IFN-α-, IFN-γ- and UVB-induced phosphorylation of STAT1 at serine 727. UVB stimulation of the keratinocytes only led to phosphorylation of STAT1 at the serine 727 site. This is in contrast to stimulation with IFN-γ, IFN-α and IL-6, which led to a phosphorylation of both the tyrosine 701 and serine 727 sites of STAT1. UVB is a known strong activator of the p38 MAPK signalling pathway, supporting the importance of this pathway in STAT1 (Ser727) phosphorylation. However, UVB is also known to have an overall immunomodulatory effect and is therefore used as a therapy for psoriasis. In that perspective it is worth noting that UVB stimulation did not result in any transcriptional activation of STAT1.

Interestingly, the IFN-α-induced phosphorylation of STAT1(Tyr701) was shown to be mediated by a PKC-**δ**-dependent mechanism, but a p38 MAPK-independent mechanism, because preincubation with rottlerin significantly reduced the IFN-α-induced phosphorylation of STAT1(Tyr701), whereas preincubation with SB202190 did not. It is possible that the observed p38 MAPK- and PKC-**δ**-dependent phosphorylation of STAT1(Ser727) reflects a PKC-**δ**-dependent activation of p38 MAPK as previously described;[18] however, further studies need to be conducted in order to clarify this.

Recent studies investigating the potential therapeutic effect of different JAK1, JAK2 and JAK3 inhibitors in psoriatic and rheumatoid arthritis models have shown very promising results.[10,11,27–29] Fridman *et al.*[10] used a small molecule inhibitor of JAK1 and JAK2, INCB018424, and showed a dose-dependent decrease in STAT3 phosphorylation. Furthermore, they were able to demonstrate suppression of oedema, lymphocyte infiltration and keratinocyte proliferation by topical treatment with INCB018424 in an IL-23- and thymic stromal lymphopoietin-induced hypersensitivity mouse model. In addition, Stump *et al.*[29] demonstrated reduced paw oedema, clinical scores and histological evidence of disease amelioration in two mouse models of rheumatoid arthritis using a small-molecule JAK2 inhibitor, CEP-33779. Based on these results, the JAK/STAT signalling pathway seems to be a promising target in the future treatment of psoriasis and other inflammatory diseases.

Taken together, this study helps to characterize further the role of the JAK/STAT signalling pathway in the pathogenesis of psoriasis and contributes to the understanding of the aetiology. Furthermore, we have provided evidence indicating that STAT1 might serve as a potential target in the treatment of psoriasis.

What's already known about this topic?

- The expression of STAT1 is increased in lesional psoriatic skin.
- STAT1 has an increased nuclear expression in psoriatic epidermis.
- STAT1 can be phosphorylated on two different phosphorylation sites: a tyrosine 701 site and a serine 727 site.
- STAT1 is involved in the regulation of interferon-responsive genes.

What does this study add?

- The phosphorylation level of STAT1(Tyr701) and STAT1(Ser727) is increased in lesional psoriatic skin.
- The two kinases, p38 mitogen-activated protein kinase and protein kinase C-**δ**, are involved in the phosphorylation of STAT1 in cultured human keratinocytes.

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Supporting Information

Additional Supporting information may be found in the online version of this article.

Fig S1. Densitometric analysis of the Western blotting band intensity from the inhibitor studies of STAT1(Tyr701) and STAT1(Ser727) phosphorylation.

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Artículo 3

Adenosine receptor expression is altered in psoriatic skin

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Letter to the Editor. Journal of Investigative Dermatology (En preparación)

Adenosine receptor expression is altered in psoriatic skin

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Abbreviations: NHK, normal human keratinocyte; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

TO THE EDITOR

Adenosine is a known endogenous purine nucleoside that regulates immunity and inflammation via occupancy of four specific cell surface receptors (A_1, A_{2A}, A_{2B} and A_3). Moreover, it mediates, at least in part, the anti-inflammatory effect of methotrexate, an immunosuppressive agent widely used to treat autoimmune inflammatory diseases such as psoriasis (Cronstein *et al.*, 1993; Chen *et al.*, 2013; Montesinos *et al.*, 2000; Morabito *et al.*, 1998).

Psoriasis is a chronic relapsing immune-mediated disease in which a permanent inflammatory state is settled upon the skin. Little is known about the effects of adenosine on epidermal cells, and reports are often contradictory. Thus, adenosine has been described to promote murine keratinocyte proliferation through activation of adenosine A_{2B} receptors (Braun *et al.*, 2006), and to inhibit human keratinocyte proliferation through its uptake by the cell membrane transporter hENT1 (Brown *et al.*, 2000). Therefore, the effects of adenosine receptor activation on inflammatory skin diseases remain unclear. Our studies are addressed to clarify the role of adenosine on the psoriatic epidermis.

For this purpose, samples from normal and psoriatic skin were obtained from foreskins of young donors and punch biopsies of lesional psoriatic skin, respectively. Psoriatic patients were newly diagnosed and biopsies were taken for histopathological analysis. Informed written consent was obtained and the study was approved by the local ethical committee and carried out according to the Declaration of Helsinki Principles. Skin samples were dispase-treated overnight and the epidermis was removed and stored at -80 °C. Expression of adenosine receptors was determined by real time RT-PCR using Taqman® probes (Applied Biosystems, Foster City, CA) following manufacturer's protocols. We confirmed that mainly adenosine A_{2B} receptors, and A_{2A} receptors to a lesser extent, are expressed in epidermis from healthy patients, while subtypes A_1 and A_3 were undetectable. Interestingly, in the epidermis from psoriatic

patients, A_{2B} receptors expression decreased by half and A_{2A} receptor expression increased two fold (Figure 1a).

Several proinflammatory stimuli are able to modulate adenosine receptor expression (Haskó *et al.*, 2009; Morello *et al.*, 2006). Therefore, we hypothesized that the change in receptor expression could be due to the inflammatory milieu present in psoriatic skin. In order to confirm this hypothesis, normal human keratinocytes (NHK) were isolated from foreskin surgical resections of healthy young donors and grown in a serum-free low-Ca^{2+} medium (Invitrogen, Carlsbad, CA), as described previously (Andres *et al.*, 2013). At 80% confluency, keratinocytes were stimulated with a series of mediators known to be augmented in psoriatic skin. After a 3h-stimulation, mRNA expression of A_{2A} and A_{2B} adenosine receptors was assessed by quantitative real time PCR. Interestingly, stimulation with IFNγ alone, and 12-O-tetradecanoylphorbol-13-acetate (TPA) to a lesser extent, reproduced the psoriatic phenotype. Additionally, TNFα and IL-1β also increased A_{2A} receptor expression, whereas IFNα decreased A_{2B} receptor expression. Other psoriatic mediators such as IL-6, IL-17 and Il-23 had no effect on adenosine receptor expression (Figure 1b).

Previous reports have shown that adenosine causes an increase in intracellular cAMP in human keratinocytes (Brown *et al.*, 2000). We incubated NHK with the non-selective adenosine agonist 5'-N-ethylcarboxamide adenosine (NECA, 10 μM), and the selective A_{2A} agonist CGS-21680 in order to assess the adenosine receptor underlying this effect. After 10 minutes, stimulation with NECA significantly increased cAMP levels whereas stimulation with CGS-21680 had no effect. Moreover, NECA-induced cAMP increase could be inhibited with the selective A_{2B} antagonist MRS 1706 whereas the A_{2A} selective antagonist SCH 442416 had no effect. These results confirm that the A_{2B} receptor is responsible for the adenosine-induced cAMP increase in NHK (Figure 2a). All agonists and antagonists were purchased from Tocris Bioscience (Bristol, UK).

Parallely, levels of TNFα and IL-8 in conditioned media were determined by ELISA after TPA for 7h. The selective A_{2A} agonist CGS-21680 dose-dependently inhibited TNFα production by stimulated keratinocytes, with similar potency to NECA. Both agonists inhibited IL-8 production but only at the highest concentration assayed (Figure 2b).

The purine nucleoside adenosine regulates a wide variety of physiologic processes via interaction with one or more of four known cell-surface receptors. Numerous studies indicate that adenosine, through the activation of its different receptor subtypes, is a potent regulator of inflammation and immunity (Haskó & Cronstein, 2004; Hasko *et al.*, 2008; Ohta & Sitkovsky, 2001). Our study demonstrates that the inflammatory mediators released during the development of a psoriatic lesion are able to modulate adenosine receptor expression. Since receptor density is a known factor affecting cellular responses to adenosine (Haskó *et al.*, 2009), this modification may modulate adenosine global effect in the epidermis. In this regard, our results show a promotion of the A_{2A} to the detriment of A_{2B} expression.

The A_{2B} is a low affinity receptor that requires pathological conditions in which adenosine concentrations are dramatically increased in order to be activated (Chen *et al.*, 2013; Haskó *et al.*, 2009). Moreover, several reports suggest that its effects are overshadowed by the higher affinity A_{2A} receptor (Hasko *et al.*, 2008). Therefore, the increase in A_{2A} expression may be of a greater impact than the decrease in A_{2B} expression in psoriatic skin. In this sense, our results demonstrate that both receptors have anti-inflammatory properties in NHK. The increase in cAMP induced by the A_{2B} receptor has a known anti-inflammatory effect that is being tested in the therapeutics of psoriasis (Schafer, 2012). On the other hand, the A_{2A} agonist CGS-21680 inhibited cytokine release in TPA-stimulated NHK. Furthermore, this receptor is one of the major contributors to adenosine anti-inflammatory effect (Ohta & Sitkovsky, 2001). Therefore, further studies will be needed in order to clarify the pathophysiological relevance of these findings and to fully understand adenosine contribution to the psoriatic phenotype.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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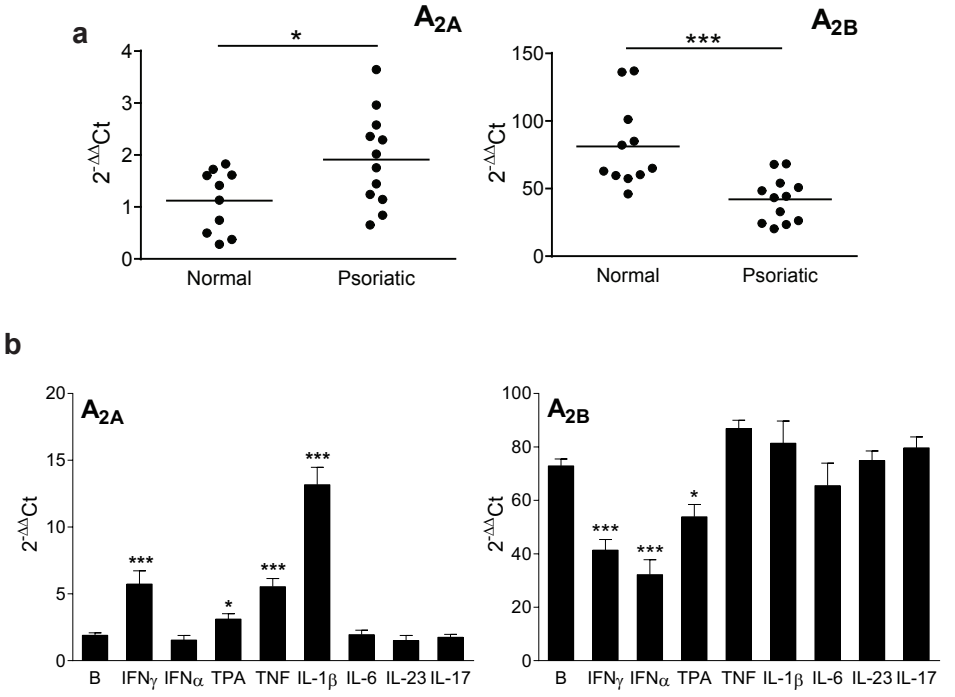

Figure 1. Adenosine receptor mRNA expression in skin and NHK. (**a**) A_{2A} and A_{2B} adenosine receptor expression in plaque type psoriasis compared to normal skin. mRNA expression, relative to housekeeping gene GAPDH, was analyzed by RT-PCR. Bars indicate mean mRNA levels ± S.E.M (n = 11 - 12). *P < 0.05; ***P < 0.001 with Student's t test. (**b**) A_{2A} and A_{2B} adenosine receptor expression in stimulated NHK. Cells were incubated with IFNγ, IFNα, (50 ng/ml), TPA (1 µg/ml), TNFα, IL-1β, IL-6, IL-23 or IL-17 (10 ng/ml) for 3 h. mRNA expression, relative to housekeeping gene GAPDH, was analyzed by RT-PCR. Bars indicate mean mRNA levels ± S.E.M (n = 3) Samples were analyzed in duplicates. *P < 0.05; ***P < 0.001 with Dunnett's t test.

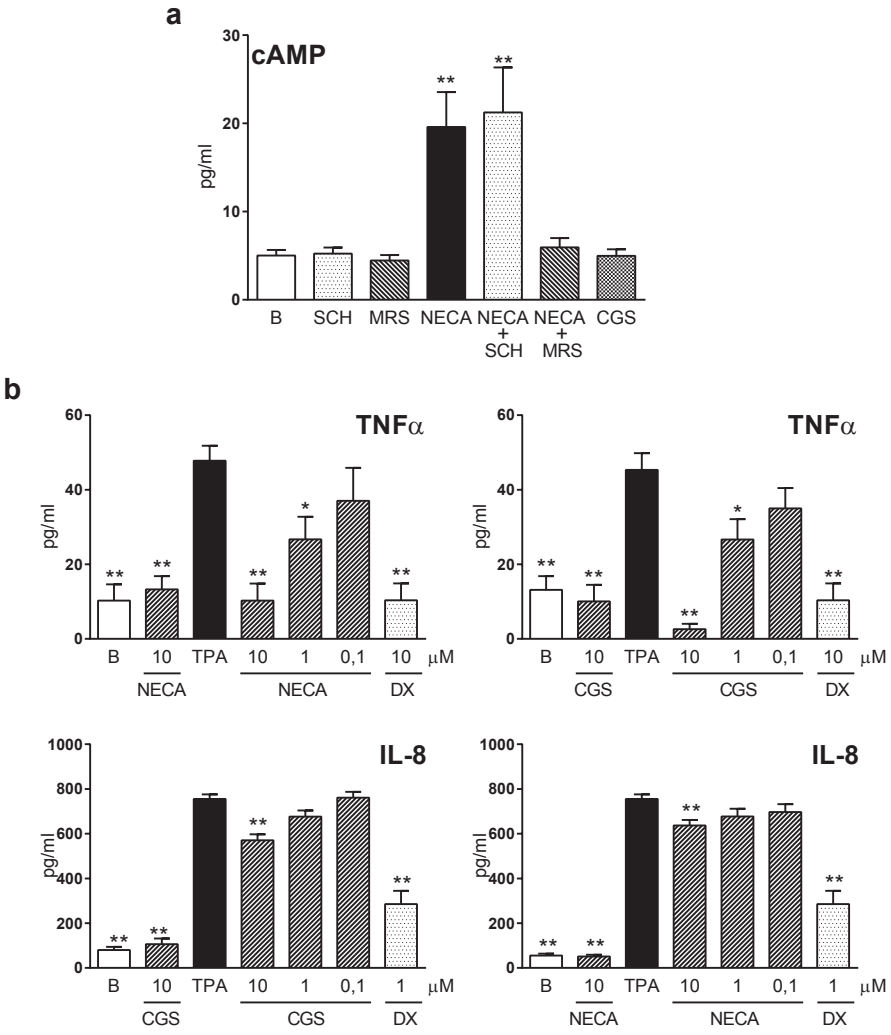

Figure 2. Activation of adenosine receptors causes an increase in cAMP and a decrease in cytokine release in NHK. (**a**) NHK were incubated with NECA (10^{-5} M) in the presence or absence of SCH 442416 (A_{2A} antagonist, 10^{-7} M) or MRS 1706 (A_{2B} antagonist, 10^{-7} M). Alternatively, cells were incubated with CGS-21680. Bars indicate mean cAMP levels ± S.E.M (*n* = 3) Samples were analyzed in triplicates. ***P* < 0.01 with Student's *t* test. (**b**) NHK were incubated with NECA or CGS-21680 at the indicated concentrations and then stimulated with TPA (1 μg/ml) for 7 h. Bars indicate mean TNFα or IL-8 levels in cell supernatants ± S.E.M (*n* = 3) Samples were analyzed in triplicates. **P* < 0.05; ***P* < 0.01 with Dunnett's *t* test.

Artículo 4

Coscinolactams A and B: new nitrogen-containing sesterterpenoids from the marine sponge Coscinoderma mathewsi exerting anti-inflammatory properties

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Coscinolactams A and B: new nitrogen-containing sesterterpenoids from the marine sponge *Coscinoderma mathewsi* exerting anti-inflammatory properties

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ABSTRACT

Investigation of the marine sponge *Coscinoderma mathewsi* led to the isolation of two novel nitrogen-containing cheilanthane sesterterpenoids, coscinolactams A and B, together with known suvanine. The structures were elucidated by extensive spectroscopic measurements including NOE experiments to deduce the stereochemistry. The natural compounds, as well as a semisynthetic derivative, showed moderate anti-inflammatory activity measured as their capability to inhibit PGE_2 and NO production. The suvanine aldehyde derivative **4** inhibited inducible nitric oxide protein expression with an IC_{50} value of 7.3 μM.

1. Introduction

Marine sponges of the *Coscinoderma* genus have been a source of sulfated terpenoids often characterized by unusual biological effects. As examples halisulfates 1[1] and 7[2] were isolated from a Micronesia collection of the sponge. Halisulfate 7 is an inhibitor of the catalytic subunits of the mammalian Ser/Thr protein phosphatases calcineurin, PP-1, and PP-2A,[3] whereas halisulfate 1 is a potent isocitrate lyase inhibitor.[4] Coscinosulfate, a sesquiterpene sulfate from the New Caledonian sponge *Coscinoderma mathewsi*, displays significant inhibitory activity towards CDC25A phosphatase.[5] The tricyclic sesterterpene suvanine[6] is a thrombin and trypsin inhibitor[1] and shows moderate antimycobacterial activity.[7]

In the course of a project directed to the chemical investigation and evaluation of the marine invertebrates of South Pacific we had the opportunity to study the sponge *C. mathewsi* Lendenfield (order Dictyoceratida, family Spongiidae) collected in Solomon Islands, whose crude ethanolic extract exhibited an anti-PLA_2 activity (72% inhibition at 400 μg/mL). The purification of the polar extracts afforded two new nitrogen-containing cheilanthane sesterterpenoids, coscinolactams A (**1**) and B (**2**), together with the known suvanine (**3**) (Fig. 1). Herein we report the isolation and structure elucidation of these compounds, as well as the preparation of two suvanine semisynthetic derivatives **4** and **5**. The in vitro pharmacological evaluation of **1** and **3**–**5** on the inhibition of four different secretary PLA_{2s} ($sPLA_2$), belonging to groups I (*Naja naja* venom and porcine pancreatic enzymes), II (human synovial recombinant enzyme) and III (bee venom enzyme), as well as on NO and PGE_2 production from macrophage line RAW 264.7, showed that the suvanine aldehyde derivative **4** exerted an interesting anti-inflammatory profile mainly through the inhibition of inducible nitric oxide expression.

2. Results and discussion

The lyophilized sponge (322 g) was extracted with MeOH, and the combined extracts were fractionated according to the Kupchan partitioning procedure.[8] The bioactive chloroform extract was purified by DCCC ($CHCl_3$/MeOH/H_2O ascending mode), followed by reverse-phase HPLC (MeOH aqueous 65%) to give suvanine (**3**).

The more polar coscinolactams A (**1**) and B (**2**) were obtained from the butanol extract chromatographed by DCCC and reverse-phase HPLC.

Coscinolactam A (**1**) was obtained as amorphous solid, $[\alpha]_D$ +25.7. The molecular formula was determined to be $C_{27}H_{41}NO_7S$ by HRESIMS (*m*/*z* 522.2547 $[M-H]^-$). The gross structure of **1** was deduced from detailed analysis of the ^1H and ^{13}C NMR spectroscopic data (Table 1) aided by 2D NMR experiments (^1H–^1H COSY, TOCSY, HSQC and HMBC). The NMR spectroscopic data showed the signals for two acyl carbons at δ 176.3 and 173.7, two trisubstituted

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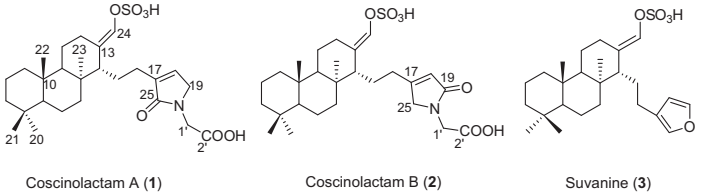


Figure 1. Cheilanthane sesterterpenoids isolated from the marine sponge *C. mathewsi*.

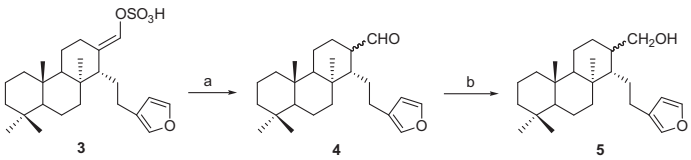
double bonds [δ_C 125.2 (s), 140.5(s), 133.9 (d), 137.8 (d); δ_H 6.89 (1H, s) and 6.36 (1H, s)] and two diastereotopic methylenes linked to a nitrogen atom [δ_H 4.00 (1H, d, J=15.9 Hz)–4.12 (1H, d, J=15.9 Hz), δ_C 52.8 and δ_H 3.89 (1H, d, J=16.9 Hz)–4.15 (1H, d, J=16.9 Hz), δ_C 46.8]. The remaining signals are at higher field and correspond to four methyls on quaternary carbons, nine methylenes, three methines and three quaternary carbons. Comparison with the NMR spectroscopic data of suvanine clearly indicated that **1** possesses the same tricyclic spongiane-like framework of suvanine. The enol sulfate functionality at C-13, characteristic of suvanine, was also present, as evidenced by ^{13}C NMR spectroscopic data and by the presence of the diagnostic ^{1}H NMR signal at δ 6.36 relative to H-24. COSY and HMBC data revealed the linkage of the C-14 to the methylene at C-15 (δ_H 1.47, 1.91, δ_C 23.6) and the allylic methylene C-16 (δ_H 2.22, 2.51, δ_C 25.9). HMBC correlations of H-18 to C-17 (δ_C 140.5), C-19 (δ_C 52.8) and C-25 (δ_C 173.7) and chemical shift of C-19 (δ_C 52.8) revealed the presence of an α,β-unsaturated γ-lactam ring (carbon atoms in **1**: C-17 to C-19, C-25 and N-25). The linkage between C-16 and C-17 was inferred by key HMBC correlation H-18 to C-16, and H_2-16 to C-25. The remaining set of resonances, a methylene (δ_H 3.89 and 4.15, δ_C 46.8) and an acyl carbonyl (δ_C 176.3), was easily assigned to a glycine residue (C-1′ to C2′). The linkage of this latter unit to N-25 was evidenced by HMBC correlations H_2-1′ to C-19 and C-25. Therefore the structure of coscinolactam A was defined as **1**. The relative stereochemistry of the tricyclic system was determined by NOESY correlations. In particular, intense NOESY cross-peaks between CH_3-22 and CH_3-21, H-12 and H-14 suggested a tricyclic system with an AB trans, BC cis stereochemistry. The stereochemistry of the exocyclic double bond was established as *E* on the basis of the observed NOE contact between H-24 (δ_H 6.36) and one of the protons belonging to diastereotopic methylene at C-15 (δ_H 1.47). The good match with the ^{1}H and ^{13}C NMR resonances of coscinolactam A with the corresponding resonances of the tricyclic portion of suvanine indirectly confirmed the proposed structure.

Coscinolactam B (**2**) was isomeric with coscinolactam A (*m*/*z* 522.2509 [M−H]$^-$). ^{1}H and ^{13}C NMR spectra of **2** were very similar to those of **1** except for the signals relative to the α,β-unsaturated γ-lactam ring. In particular the upfield shift of the olefinic proton (δ_H 5.85 in **2** vs 6.89 in **1**) and the downfield shift of the γ-carbon (δ_C 56.3 in **2** vs 52.8 in **1**) suggested the presence of a β-substituted α,β-unsaturated γ-lactam ring. The linkage of the β-carbon of the lactam ring to C-16 was also confirmed by the diagnostic HMBC correlation between H_2-16 and C-25.

Table 1
^{1}H and ^{13}C NMR spectroscopic data (700 MHz, CD_3OD) for coscinolactams A (**1**) and B (**2**)

	1			**2**		
	δ_H[a]	δ_C	HMBC	δ_H[a]	δ_C	HMBC
1	1.86 ovl, 0.87 dd (14.0, 7.4)	43.2		1.86 ovl, 0.87	43.1	
2	1.63 ovl, 1.40 ovl	19.7		1.62 ovl, 1.40 ovl	19.6	
3	1.40 ovl, 1.16 m	43.0		1.40 ovl, 1.16 m	43.0	
4	—	34.3		—	34.0	
5	1.06 dd (9.0, 4.4)	54.2	C1, C4, C6, C10, C21,	1.08 dd (11.2, 4.6)	54.1	C1, C4, C6, C21
6	1.54 m, 1.33 ovl	19.3		1.60 ovl, 1.34 ovl	19.3	
7	1.90 ovl, 1.33 ovl	36.3	C5, C6, C8, C9, C23	1.90 ovl, 1.37 ovl	36.1	
8	—	39.9		—	39.7	
9	0.91 t (7.6)	58.6	C1, C8, C11, C12, C22, C23	0.92 t (6.0)	58.3	C1, C8, C11, C12, C22, C23
10	—	40.2		—	40.1	
11	1.70 m, 1.61 ovl	20.9		1.71 m, 1.62 ovl	20.9	
12	2.60 dt (15.9, 6.1), 2.18 m	25.4	C9, C11, C13, C14, C24	2.59 m, 2.18 dt (16.2, 8.5)	25.2	C9, C11, C13, C14, C24
13	—	125.2		—	124.9	
14	2.35 d (10.4)	43.9	C8, C13, C15, C16, C23, C24	2.33 d (10.4)	43.9	C8, C13, C15, C16, C23, C24
15	1.91 ovl, 1.47 m	23.6		1.89 ovl, 1.57 ovl	23.7	
16	2.51 m, 2.22 m	25.9	C14, C15, C17, C18, C25	2.62 m, 2.36 dt (16.1, 8.1)	29.1	C14, C15, C17, C18, C25
17	—	140.5		—	164.2	
18	6.89 s	137.8	C16, C17, C19, C25	5.85 s	121.3	C16, C17, C19, C25
19	4.12 d (15.9), 4.00 d (15.9)	52.8	C17, C18	—	174.0	
20	0.86 s	33.7	C3, C4, C5, C21	0.87 s	33.8	C3, C4, C5, C21
21	0.89 s	22.2	C3, C4, C5, C20	0.89 s	22.1	C3, C4, C5, C20
22	1.05 s	18.5	C1, C5, C9, C10	1.05 s	18.5	C1, C5, C9, C10
23	0.84 s	26.5	C7, C8, C9, C14	0.86 s	26.5	C7, C8, C9, C14
24	6.36 s	133.9	C12, C13, C14	6.27 s	133.3	C12, C13, C14
25	—	173.7		4.18 d (16.8), 4.20 d (16.8)	56.3	C17, C18, C19,
1′	4.15 d (16.9), 3.89 d (16.9)	46.8	C19, C25, C2′	3.82 d (17.1), 3.84 d (17.1)	39.5	C19, C25
2′	—	176.3		—	n.o	

Ovl: overlapped.
[a] Coupling constants are in parentheses and given in hertz. ^{1}H and ^{13}C assignments aided by COSY, TOCSY, HSQC and HMBC experiments.



Scheme 1. (a) dioxane/pyridine, 180 °C; (b) $NaBH_4$, MeOH, rt.

Table 2
Inhibitory activity (% I) of compounds **1** and **3–5** at 10 μM on different $sPLA_2$ belonging to the groups IIA, IA (*Naja naja* venom), IB (porcine pancreatic enzyme) and III (bee venom enzyme)

Compound (10 μM)	GroupIIA-$sPLA_2$, % inhibition	GroupIA-$sPLA_2$, % inhibition	GroupIB-$sPLA_2$, % inhibition	GroupIII-$sPLA_2$, % inhibition
1	6.7±1.6	0.0±0.0	0.0±0.0	0.0±0.0
3	32.8±1.3**	0.0±0.0	0.0±0.0	29.4±3.0**
4	10.6±5.3	0.0±0.0	0.0±0.0	0.0±0.0
5	7.6±4.0	0.0±0.0	0.0±0.0	0.0±0.0
LY311727	96.3±1.7**	7.9±5.6	36.9±11.0**	2.4±1.8

Results show mean±S.E.M. (n=6). Statistical significances: **$p<0.01$, with respect to the corresponding enzyme control group (IIA $sPLA_2$=12,129±384 cpm; IA $sPLA_2$=10,973±350 cpm; IB $sPLA_2$=8008±47 cpm; III $sPLA_2$=14,854±1054 cpm). Enzyme control group contains the vehicle (ethanol 1%). LY311727 used as control.

Whereas lactam systems are quite unusual in natural products, a similar system consisting of an aminoacyl-derived unit fused with a terpenoid system was found in echinophyllins A[9] and D[10] clerodane diterpenoids from the Brazilian medicinal plant *Echinodorus macrophyllus*. ^1H and ^{13}C NMR spectroscopic data of coscinolactams A and B match very well with those of echinophyllins A and D relative to the isomeric γ-lactam ring subunits. A glycine-derived lactam system was found in spongolactam C, a diterpene derivative isolated from the Okinawan marine sponge *Spongia* sp.[11]

Table 3
Inhibitory activity of compounds **1** and **3–5** at 10 μM on the production of PGE_2 and NO in LPS-stimulated RAW 264.7 cells

Compound (10 μM)	PGE_2 % inhibition	NO % inhibition
1	48.5±3.0**	31.8±4.0**
3	41.6±4.6**	21.0±5.7
4	49.2±4.1**	60.0±8.1**
5	14.9±2.1**	31.9±2.8**
Dexamethasone (1 μM)	78.7±5.4**	96.6±1.4**

Results show mean±SEM (n=6). Statistical significances: **$p<0.01$, with respect to the LPS-stimulated control group (contains the vehicle ethanol 1%). PGE_2 (non-stimulated cells=0.6±0.2 ng/mL; LPS-stimulated cells=16.0±1.6 ng/mL). Nitrite (non-stimulated cells=48.8±3.6 ng/mL; LPS-stimulated cells=414.1±16.4 ng/mL). Dexamethasone used as control.

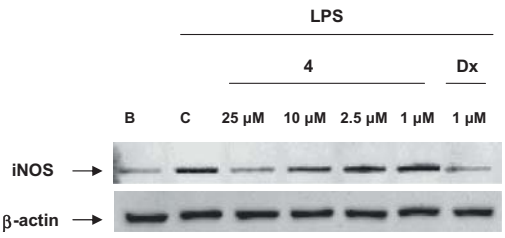


Figure 2. Effect of compound **4** on iNOS expression in LPS-stimulated RAW 264.7 cells. The figure is representative of two similar experiments; B: normal cells; C: LPS-stimulated cells. Dx=Dexamethasone.

Biogenetically the isomeric coscinolactams A and B may be derived from suvanine, through oxidation to isomeric γ-hydroxybutenolide derivatives, condensation with glycine and reduction to γ-lactam, which has been chemically explored in the semisynthesis of spongolactam C from the corresponding furanoterpene.[11]

To investigate the role of the sulfate group on the pharmacological profile of these natural compounds, major suvanine was subjected to hydrolytic and reductive transformations depicted in Scheme 1.

Compounds **1** and **3–5** were tested against four different secretory PLA_2 (belonging to groups IA, IB, IIB and III) (Table 2). LY311727, a well known inhibitor of group IIA $sPLA_2$, was used as a reference tool.[12] Despite the good activity previously exhibited by the crude extract, only suvanine showed a moderate activity against human synovial $sPLA_2$-IIA and bee venom $sPLA_2$-III. In addition, compounds **1** and **3–5** were devoid of significant cytotoxic effects on RAW 264.7 at concentrations up to 10 μM, as assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (data not shown). Their ability to inhibit PGE_2 and NO production on RAW 264.7 cells stimulated with LPS was also investigated (Table 3). Interestingly, the suvanine aldehyde derivative **4** showed an improved capability to inhibit NO production and to a lesser extent PGE_2, whereas the suvanine alcoholic derivative **5** was proved to be almost inactive in all experiments. Compound **4** inhibited dose-dependently the production of NO, showing an IC_{50} value of 7.3±2.3 μM. The NO reduction was the consequence of the inhibition of the expression of inducible NO synthase (Fig. 2), exactly as the dexamethasone control drug did. This suvanine aldehyde derivative **4**, which can mainly reduce NO and PGE_2 production by affecting the expression of the inducible NO synthase enzyme, could be an interesting strategy to obtain promising anti-inflammatory agents.

3. Experimental

3.1. General experimental procedures

Specific rotations were measured on a Perkin–Elmer 243 B polarimeter. High-resolution ESI-MS spectra were performed with a Micromass QTOF Micromass spectrometer. ESI-MS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. NMR spectra were obtained on Varian Inova 500 and Varian Inova 700 NMR spectrometers (^1H at 500 and 700 MHz, ^{13}C at 125 and 175 MHz, respectively) equipped with a Sun hardware, δ (ppm), J in Hz, spectra referred to CD_3OD as internal standards (δ_H=3.31). HPLC was performed using a Waters Model 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401.

All reagents were commercially obtained (Aldrich, Fluka) at the highest commercial quality and used without further purification except where noted. All reactions were monitored by TLC on silica gel plates (Macherey–Nagel).

3.2. Sponge material and separation of individual sesterterpenoids

C. mathewsi Lendenfield (order Dictyoceratida, family Spongiidae) was collected on the barrier reef of Vangunu Island, Solomon Islands, in July 2004. The samples were frozen immediately after collection and lyophilized to yield 322 g of dry mass. The sponge was identified by Dr. John Hooper, Queensland Museum, Brisbane, Australia, where a voucher specimen is deposited under the accessing number G322695.

The lyophilized material (322 g) was extracted with methanol (3×3 L) at room temperature and the crude methanolic extract (72.6 g) was subjected to a modified Kupchan's partitioning procedure as follows. The methanol extract was dissolved in a mixture of MeOH/H_2O containing 10% H_2O and partitioned against *n*-hexane. The water content (% v/v) of the MeOH extract was adjusted to 30% and partitioned against $CHCl_3$. The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH. The hexane, chloroform and butanol extracts were tested against PLA_2 at 400 mg/mL dose showing 94, 93 and 59% inhibition, respectively.

The *n*-BuOH extract (4 g) was chromatographed in four runs by DCCC ($CHCl_3$/MeOH/H_2O, 7:13:8, ascending mode) and fractions of 6 mL were collected and combined on the basis of their similar TLC retention factors.

Fraction 7 (28.4 mg) was purified by HPLC on a Nucleodur C18 column (3 μm, 150×4.60 mm, flow rate 1.0 mL/min) with 45% MeOH/H_2O as eluent to give 3.1 mg of coscinolactam A (**1**) (t_R=10 min) and 1.9 mg of coscinolactam B (**2**) (t_R=11 min).

The chloroform-soluble material (1.5 g), mainly containing suvanine, was chromatographed by DCCC using $CHCl_3$/MeOH/H_2O (7:13:8) in the ascending mode (the lower phase was the stationary phase); the flow rate was 18 mL/h; 6 mL fractions were collected and combined on the basis of their similar TLC retention factors.

Fraction 2 (262 mg) was purified by HPLC on a μ-Bondapack C18 column (10 μm, 300×7.8 mm, flow rate 4.0 mL/min) with 65% MeOH/H_2O as eluent to give 240 mg of suvanine (t_R=8 min).

3.3. Characteristic data for each compound

Coscinolactam A: white amorphous solid; $[\alpha]_D^{25}$ +25.7 (*c* 0.07, methanol); ^1H and ^{13}C NMR data in CD_3OD given in Table 1; ESI-MS: *m*/*z* 522.3 $[M-H]^-$. HRMS (ESI): calcd for $C_{27}H_{40}NO_7S$: 522.2525; found 522.2547 $[M-H]^-$.

Coscinolactam B: white amorphous solid; $[\alpha]_D^{25}$ +8.57 (*c* 0.07, methanol); ^1H and ^{13}C NMR data in CD_3OD given in Table 1; ESI-MS: *m*/*z* 522.3 $[M-H]^-$. HRMS (ESI): calcd for $C_{27}H_{40}NO_7S$: 522.2525; found 522.2509 $[M-H]^-$.

Suvanine: white amorphous solid; $[\alpha]_D^{25}$ +12.2 (*c* 0.4, methanol); ESI-MS: *m*/*z* 522.3 $[M-H]^-$. HRMS (ESI): calcd for $C_{25}H_{37}O_5S$: 449.2362; found 449.2387 $[M-H]^-$.

3.4. Synthetic procedures

3.4.1. Aldehyde **4**

A solution of suvanine **3** (15 mg, 0.033 mmol) in pyridine (0.5 mL) and dioxane (0.5 mL) was heated at 150 °C for 2.5 h in a stoppered reaction vial. After the solution was cooled, the mixture was evaporated to dryness and then purified by HPLC on a μ-Bondapak C18 column (30 cm×3.9 mm i.d.) with MeOH/H_2O 80:20, to give aldehyde **4** as a 9:1 mixture of two diastereoisomers (9.2 mg, 80%). ESI-MS: 371.30 $[M+H]^+$; selected ^1H NMR (500 MHz, $CDCl_3$) for major diastereoisomer: 9.48 (1H, s, –CHO), 7.39 (1H, s), 7.28 (1H, s), 6.33 (CH-18, s), 1.04 (3H, s), 0.87 (3H, s), 0.86 (3H, s), 0.84 (3H, s).

3.4.2. Alcohol **5**

$NaBH_4$ was added in one portion to a stirred solution of **4** (5.2 mg, 0.014 mmol) in MeOH at room temperature. The mixture was stirred for 1 h and then was concentrated in vacuo and then diluted with NH_4Cl and ethyl acetate. The aqueous phase was extracted with ethyl acetate and then the combined organic extracts were washed with brine, dried (Na_2SO_4) and concentrated in vacuo to yield alcohol **5** as a 9:1 mixture of two diastereoisomers (4.7 mg, 90%). ESI-MS: 373.40 $[M+H]^+$; selected ^1H NMR (500 MHz, $CDCl_3$) for major diastereoisomer: 7.39 (1H, s), 7.28 (1H, s), 6.33 (1H, s), 3.71 (d, *J*=11.3 Hz, H-24), 3.43 (dd, *J*=11.3, 4.5 Hz, H-24), 1.13 (3H, s), 0.90 (3H, s), 0.86 (6H, s).

3.5. Anti-inflammatory assays

3.5.1. Materials

[5,6,8,11,12,14,15(*n*)-^3H] PGE_2 and [9,10-^3H]oleic acid were purchased from Amersham Biosciences (Barcelona, Spain). Inducible NO synthase specific polyclonal antiserum was purchased from Cayman Chem. (MI, USA). The peroxidase-conjugated goat anti-rabbit Immunoglobulin G (IgG) was purchased from Dako (Copenhagen, Denmark). The rest of reagents was from Sigma (MO, USA).

3.5.2. Assay of $sPLA_2$

$sPLA_2$ activity was assayed using [^3H]-oleate labelled membranes of *Escherichia coli*, following a modification of the method of Franson et al.[13,14] *E. coli* strain CECT 101 was grown for 6–8 h at 37 °C in the presence of 5 μCi/mL [^3H]-oleic acid (specific activity 10 Ci/mmol) until the end of the logarithmic phase. After centrifugation at 1800*g* for 10 min at 4 °C, the membranes were washed, resuspended in PBS and autoclaved for 30–45 min. At least 95% of the radioactivity was incorporated into the phospholipid fraction. *Naja naja* venom (Group IA $sPLA_2$), porcine pancreatic (Group IB $sPLA_2$), human recombinant synovial (Group IIA $sPLA_2$), and bee venom (Group III $sPLA_2$) enzymes were used as sources of $sPLA_2$. Enzymes were diluted in 10 μL of 100 mM Tris–HCl, 1 mM $CaCl_2$ buffer pH 7.5 and preincubated at 37 °C for 5 min with test compound or vehicle in a final volume of 250 μL. Incubation proceeded for 15 min in the presence of 20 μL of [^3H]oleic-*E. coli* membranes and was terminated by addition of 100 μL ice-cold solution of 0.25% BSA in saline to a final concentration of 0.07% w/v. After centrifugation at 2500×*g* for 10 min at 4 °C, the radioactivity in the supernatants was determined by liquid scintillation counting.

3.5.3. Culture of murine macrophage RAW 264.7

The mouse macrophage cell line RAW 264.7 (Cell Collection, Department of Animal Cell Culture, C.S.I.C., Madrid, Spain) was cultured in Dulbecco's modified Eagle's medium (DMEM) containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% foetal bovine serum. Cultures were maintained at 37 °C in 5% CO_2 (air: CO_2, 95:5) humidified incubator. Cells were resuspended at a concentration of 1.5×10^6 cells/mL.

3.5.4. Nitric oxide and PGE_2 production in RAW 264.7 macrophages

RAW 264.7 macrophages (1.5×10^6 cells/mL) were co-incubated in 96-well culture plate (200 μL) with 1 μg/mL of *E. coli* [serotype 0111:B4] lipopolysaccharide (LPS) at 37 °C for 20 h in the presence of test compounds or vehicle. Nitrite concentration as reflection of NO release was assayed fluorometrically.[15] PGE_2 levels were determined in culture supernatants by radioimmunoassay.[16] The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan[17] was used to assess the possible cytotoxic effects of compounds (100% viability=0.533±0.010 at 492 nm).

3.5.5. Western blot assay of iNOS

Cellular lysates from RAW 264.7 (murine macrophages 1.5×10^6 cell/mL) incubated for 18 h with LPS (1 μg/mL) were obtained with lysis buffer A (10 mM HEPES, pH 8.0, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 5 mM NaF, 1 mM Na_3VO_4, 10 mM Na_2MoO_4, 1 μg/mL leupeptin, 0.1 μg/mL aprotinin and 0.5 mM phenylmethyl sulfonyl fluoride). Following centrifugation (10,000×*g*/15 min/4 °C), supernatant protein was determined by the Bradford method[18] using bovine serum albumin (BSA) as standard. iNOS protein expression was studied in the total fraction. Equal amounts of protein (25 μg) were loaded on 15% SDS-PAGE and transferred onto polyvinylidene difluoride membranes for 90 min at 125 mA. Membranes were blocked in PBS (0.02 M pH 7.0)–Tween 20 (0.1%), containing 3% w/v unfatted milk and incubated with specific polyclonal antibody against iNOS (1/1000). Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1/10,000). The immunoreactive bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences, Barcelona, Spain).

3.5.6. Statistical analysis

The results are presented as mean±SEM, which represents the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations (n=6). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.[19]

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Artículo 5

Toward the Discovery of New Agents Able to Inhibit the Expression of Microsomal Prostaglandin E Synthase-1 Enzyme as Promising Tools in Drug Development

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Research Article

Toward the Discovery of New Agents Able to Inhibit the Expression of Microsomal Prostaglandin E Synthase-1 Enzyme as Promising Tools in Drug Development

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In our recent studies, we focused our attention on the synthesis of several γ-hydroxybutenolides designed on the basis of petrosaspongiolide M 1 (PM) structure that has been recognized to potently inhibit the inflammatory process through the selective PLA_2 enzyme inhibition. By means of a combination of computational methods and efficient synthetic strategies, we generated small collections of PM modified analogs to identify new potent PLA_2 inhibitors, suitable for clinical development. In the course of the biological screening of our compounds, we discovered a potent and selective inhibitor of *m*PGES-1 expression, the benzothiophene γ-hydroxybutenolide 2, which so far represents the only product, together with resveratrol, able to reduce PGE_2 production through the selective downregulation of mPGES-1 enzyme. In consideration that microsomal prostaglandin E synthase 1 (*m*PGES-1) is one of the most strategic target involved both in inflammation and in carcinogenesis processes, we decided to explore the biological effects of some structural changes of the γ-hydroxybutenolide 2, hoping to improve its biological profile. This optimization process led to the identification of three strictly correlated compounds 14g, 16g, and 18 with higher inhibitory potency on PGE_2 production on mouse macrophage cell line RAW264.7 through the selective modulation of *m*PGES-1 enzyme expression.

Key words: COX-2, *m*PGES-1, PGE_2, γ-Hydroxybutenolide derivatives

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In the last few years, we have been involved in the discovery of new lead candidates suitable for the development of promising anti-inflammatory agents. Our research work was based on a sesterterpene marine metabolite, petrosaspongiolide M (PM) **1**, which was proved to potently inhibit the human synovial PLA_2 type IIA enzyme, responsible for the triggering of the inflammation pathway (1). Encouraged by its interesting biological profile, PM (Figure 1) was selected by our research group for extensive investigations in terms of chemical, synthetical, and biological aspects. In fact, computational methods allowed us to propose molecular details of its mode of interaction with the target enzyme (2). The high densely functionalized scaffold, represented by the γ-hydroxybutenolide moiety, forced us to find efficient synthetic strategies to generate focused collections of simplified analogs of the parent molecule suitable for a structure–activity relationship study. Despite we did not find for the synthetic molecules satisfying level of activity toward our selected target, PLA_2 enzyme, nevertheless we had the chance to discover a very interesting product, the benzothiophene γ-hydroxybutenolide **2**, which has been proved to be a potent and selective modulator of *m*PGES-1 expression (3,4). This downstream PG synthesizing enzyme, glutathione dependent, is of inducible type and that is one of the reasons why it has established itself as a novel drug target in the areas of inflammation (5,6), atherosclerosis (7), stroke (8), and cancer (9–11). On the basis of these premises, we decided to rely on some well-reasoned structural changes of the basic molecule **2**, in the attempt to improve its pharmacological behavior. For this purpose, we synthesized a series of analogs closely related with the leader compound **2**, whose biological activity was also investigated (Figure 2).

Methods and Materials

General methods

All water and air sensitive reactions were carried out under an inert atmosphere (N_2) in oven- or flame-dried glassware. Ethyl acetate, dichloromethane (DCM) and tetrahydrofuran (THF) were distilled from CaH_2 immediately prior to use. Water was degassed under vacuum (10 mbar). All reagents were used from commercial sources without any further purification. Organic extracts were dried over anhydrous Na_2SO_4. Reactions were monitored on silica gel 60 F254 (Merck) plates and visualized with potassium permanganate, cerium sulfate or ninhydrin and under UV (λ = 254 nm,

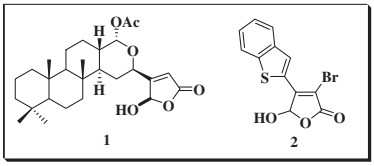


Figure 1: Petrosaspongiolide M (**1**) and 4-benzo[b]thiophen-2-yl-3-bromo-5-hydroxy-5H-furan-2-one (**2**).

365 nm). Flash column chromatography was performed using Merck 60/230–400 mesh silica gel. Analytical and semi-preparative reverse-phase HPLC purifications were performed on a Waters instrument using Jupiter C-18 column (250 × 4.60 mm, 5 μm, 300 Å; 250 × 10.00 mm, 10 μm, 300 Å, respectively). Purity grade of final products was determined on a Agilent 1100 HPLC using two different analytical reverse-phase columns (Method A: Jupiter C-18, 250 × 4.60 mm, 5 μm, 300 Å; Method B: Jupiter C-4, 250 × 4.60 mm, 5 μm, 300 Å). Reaction yields refer to chromatographically and spectroscopically pure products. Proton-detected (1H, HMBC, HSQC) and carbon-detected NMR spectra were recorded on Bruker instruments of Avance series operating at 300 and 600 MHz and 75 and 150 MHz, respectively. Chemical shifts are expressed in parts per million (ppm) on the delta (δ) scale. The solvent peak was used as internal reference: for 1H NMR $CDCl_3$ = 7.26 ppm; for ^{13}C NMR: $CDCl_3$ = 77.0 ppm. Multiplicities are reported as follows: s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets. High-resolution mass spectra (HRMS) were recorded on a Q/TOF Premier WATERS (Milford, MA, USA) mass spectrometer using an electrospray ion source (ESI-MS).

[5,6,8,11,12,14,15(*n*)-^{3}H] PGE_2 and [9,10-^{3}H]oleic acid were purchased from Amersham Biosciences (Barcelona, Spain). The rest of reagents were from Sigma (St. Louis, MO, USA). *Escherichia coli* strain CECT 101 was a gift from Professor Uruburu, Department of Microbiology, University of Valencia, Spain.

Synthesis of 3,4-dibromo-5-(2-methoxy-ethoxymethoxy)-5H-furan-2-one (4)

Mucobromic acid **3** (100 mg, 0.387 mmol) was dissolved in 10 mL of dry dichloromethane, and MEM-Cl (66 μL, 0.581 mmol) was added to the solution. Diisopropylethylamine (101 μL, 0.581 mmol) was added dropwise over a period of 15 min. After 4 h, the reaction mixture was quenched with 20 mL of HCl 1 M. The aqueous layer was extracted with dichloromethane (3 × 30 mL), and the organics were dried with Na_2SO_4, filtered and concentrated in vacuo affording dark oil. The crude oil was purified by flash chromatography (10% diethyl ether/hexane) to give **4** (115 mg, 86% yield): ^{1}H NMR δ (300 MHz; $CDCl_3$): δ 6.10 (1H, s, OC*H*O), 5.20 (1H, d, *J* = 7 Hz, OC*H*HO), 4.87 (1H, d, *J* = 7 Hz, OCH*H*O), 3.79 (1H, m, OC*H*HCH$_2$O), 3.40 (3H, s, OC*H*$_3$), 3.60 (1H, m, OCH*H*CH$_2$O), 3.54 (2H, dd, OCH$_2$C*H*$_2$O); ^{13}C NMR δ (75 MHz; $CDCl_3$): δ 167.4, 144.2, 119.1, 100.4, 95.2, 72.1, 69.0, 59.8; HRMS calcd. for $C_8H_{11}Br_2O_5$: $[M+H]^+$ 344.8973, 346.8953, 348.8932 (ratio 1:2:1); found $[M+H]^+$ 344.8942, 346.8925, 348.8918 (ratio 1:2:1).

Esterification of boronic acid 7 and 8

The boronic acids **7** and **8** (0.667 mmol) were dissolved in 6 mL of ethyl acetate, and under stirring, pinacol (0.667 mmol) was added. After 4 h, the reaction was stopped adding anhydrous Na_2SO_4 (1 g) and $CaCl_2$ (1 g). The mixture was filtered and concentrated in *vacuo* (yield: 90% of **9** and 92% of **10**).

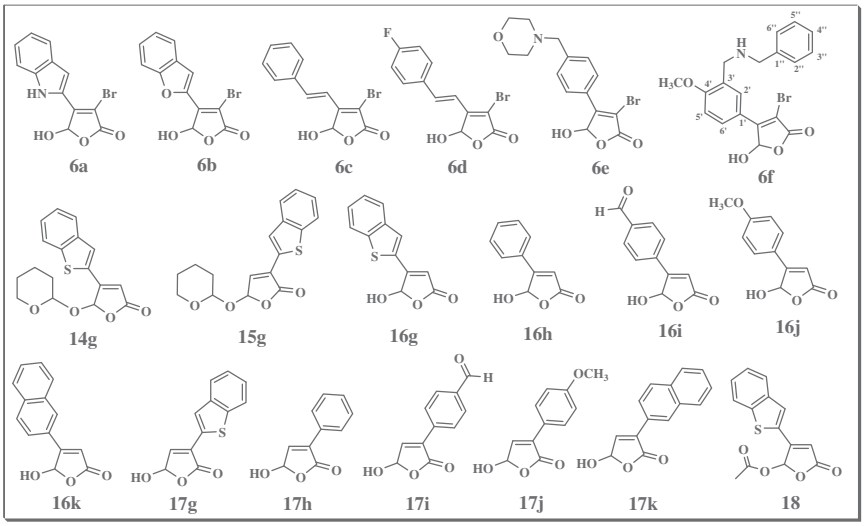


Figure 2: Collection of synthesized compounds.

Reductive amination: general procedure

Under inert atmosphere (N_2), 1 equiv. of boronic ester **9** or **10** was dissolved in anhydrous methanol (1 mL/0.18 mmol of ester). The mixture was kept under stirring at room temperature; anhydrous amine **11** or **12** (4 equiv.), $ZnCl_2$ (0.5 equiv.), and $NaCNBH_3$ (1 equiv.) were added. After 4 h, when the reagents disappeared, the reaction was stopped and 10 mL of an aqueous solution of NaOH 0.1 M was added. After concentration of methanol in *vacuo*, the aqueous phase was extracted with ethyl acetate (3 × 10 mL) and the organics were dried over Na_2SO_4, filtered and concentrated in *vacuo*.

The obtained oil was purified on silica gel by flash chromatography (10% ethyl acetate/n-hexane; yield: 79% of **e**).

BOC_2O protection of amine 13

At 0 °C, 1 equiv. of compound **13** was dissolved in a mixture of triethylamine/methanol 1:7 (v/v). The reaction was stirred for 10 min, and then a solution of BOC_2O (1.5 equiv.) was added dropwise. After 1 h, the ice bath was removed and the reaction was kept on stirring at room temperature overnight. Finally, the reaction material was first concentrated in *vacuo* and then dissolved in 20 mL of dichloromethane and 20 mL of distilled water. The aqueous phase was extracted with dichloromethane (3 × 20 mL); the organics were dried over Na_2SO_4, filtered and concentrated in *vacuo* (yield: 57% of **N-BOC-f**).

Microwave-assisted Suzuki coupling: general procedure

Compound **4** (1 equiv.), the boronic ester (**e** or **N-BOC-f**) or boronic acid **a-d** (1.5 equiv.), $Pd(dppf)_2Cl_2$ (0.03 equiv.), TBAB (0.5 equiv.), and CsF (4 equiv.) were placed in a CEM Discover vial. Water (500 μL) and THF (500 μL) were added under argon. The mixture was irradiated for 3–6 min, setting the power at 200 W, the temperature at 120 °C, the pressure at 250 psi, and the Power Max ON. After diluting (10 mL) with dichloromethane (DCM), 10 mL of an aqueous solution of HCl 1 N was added. The aqueous layer was extracted with DCM (3 × 10 mL). The organics were then dried over Na_2SO_4, filtered and concentrated in *vacuo*. The crude was purified by flash chromatography (from 10% diethyl ether/n-hexane to 40% diethyl ether/n-hexane), yield: 40–60%.

General procedure for MEM cleavage

The protected compounds **5a-e and N-BOC-f** were dissolved in a solution of trifluoroacetic acid (95%), triisopropylsilan (2.5%), and water (2.5%). The mixture was stirred at room temperature for 1.5 h and concentrated in *vacuo* at the end of the reaction.

General procedure for acetylation of 16g

Compound **16g** was dissolved in dichloromethane, the mixture was stirred at room temperature and then acetic anhydride (5 equiv.) and diisopropylethylamine (5 equiv.) were added. After 2 h, the mixture was diluted with 10 mL of HCl 1 N and the aqueous phase was extracted with dichloromethane (3 × 10 mL). The organics were dried over Na_2SO_4, filtered and concentrated *in vacuo*.

3-Bromo-5-hydroxy-4-(1H-indol-2-yl)-5H-furan-2-one (6a)

Yield: 90%; ^{1}H-NMR δ (300 MHz; $CDCl_3$): 9.25 (1H, s, N*H*), 7.72 (1H, d, *H*-8′), 7.46 (2H, m, *H*-3′, *H*-7′), 7.38 (1H, t, *H*-6′), 7.20 (1H, d, *H*-5′), 6.48 (1H, s, OC*H*OH); ^{13}C-NMR δ (75 MHz; $CDCl_3$): 166.7, 149.1, 139.6, 135.5, 134.8, 129.1, 124.0, 124.4, 124.3, 122.9, 112.0, 98.1; HRMS calcd. for $C_{12}H_7BrNO_3$: $[M - H]^-$ 293.1 and 291.1 (1:1); found 293.1 and 291.1 (1:1).

4-Benzofuran-2-yl-3-bromo-5-hydroxy-5H-furan-2-one (6b)

Yield: 88%; ^{1}H-NMR δ (300 MHz; $CDCl_3$): 7.74 (1H, s, *H*-3′), 7.73 (1H, d, *H*-8′), 7.59 (1H, d, *H*-5′), 7.47 (1H, t, *H*-7′), 7.35 (1H, t, *H*-6′), 6.55 (1H, s, OC*H*OH); ^{13}C-NMR δ (75 MHz; $CDCl_3$): 166.8, 151.1, 141.2, 137.0, 135.8, 130.8, 126.2, 125.8, 124.6, 124.1, 113.2, 99.1; HRMS calcd. for $C_{12}H_6BrO_4$: $[M - H]^-$ 294.1 and 292.1 (1:1); found 294.1 and 292.1 (1:1).

3-Bromo-5-hydroxy-4-styryl-5H-furan-2-one (6c)

Yield: 89%; ^{1}H-NMR δ (300 MHz; $CDCl_3$): 7.59 (3H, m, *H*-3′, *H*-4′, *H*-5′), 7.42 (2H, d, *H*-2′, *H*-6′), 7.38 (1H, d, *H*-1), 6.98 (1H, d, *H*-2), 6.34 (1H, s, OC*H*OH); ^{13}C-NMR δ (75 MHz; $CDCl_3$): 166.3, 156.3, 136.2, 130.5, 130.4, 130.2, 129.7, 128.2, 128.1, 127.1, 107.1, 103.8; HRMS calcd. for $C_{12}H_8BrO_3$: $[M - H]^-$ 280.1 and 278.1 (1:1); found 280.1 and 278.1 (1:1).

3-Bromo-4-[2-(4-fluoro-phenyl)-vinyl]-5-hydroxy-5H-furan-2-one (6d)

Yield: 88%; ^{1}H-NMR δ (300 MHz; $CDCl_3$): 7.58 (2H, d, *H*-3′, *H*-5′), 7.36 (1H, d, *H*-1), 7.11 (2H, d, *H*-2′, *H*-6′), 6.90 (1H, s, *H*-2), 6.34 (1H, s, OC*H*OH); ^{13}C-NMR δ (75 MHz; $CDCl_3$): 166.5, 103.8, 156.7, 107.6, 127.9, 130.8, 132.5, 130.1, 117.1, 163.5; HRMS calcd. for $C_{12}H_7BrFO_3$: $[M - H]^-$ 298.1 and 296.1 (1:1); found 298.1 and 296.1 (1:1).

3-Bromo-5-hydroxy-4-(3-morpholin-4-ylmethyl-phenyl)-5H-furan-2-one (6e)

Yield: 93%; ^{1}H-NMR δ (600 MHz; MeOD): 8.12 (1H, d, *H*-6′), 8.10 (1H, d, *H*-2′), 7.73–7.68 (2H, d, *H*-3′, *H*-5′), 6.62 (1H, s, OC*H*OH), 4.46 (2H, s, -CH_2N), 3.99–3.81 (4H, m, O-(CH_2)$_2$), 2.99 (2H, m, N-CH_2), 2.85 (2H, m, N-CH_2); ^{13}C-NMR (150 MHz, MeOD): 166.6, 155.7, 138.1, 133.2, 130.8, 130.4, 129.7, 129.3, 110.4, 98.8, 69.2, 69.1, 56.6, 56.4, 51.3; ESI-MS calcd. for $C_{15}H_{17}BrNO_4$: $[M+H]^+$ 354.0 and 356.0 (1:1); found 354.1 and 356.1 (1:1).

4-[3-(Benzylamino-methyl)-4-methoxy-phenyl]-3-bromo-5-hydroxy-5H-furan-2-one (6f)

Yield: 97%; ^{1}H-NMR δ (300 MHz; MeOD): 8.20 (1H, d, *H*-6′), 8.05 (1H, s, *H*-2′), 7.49 (5H, m, *H*-2″, *H*-3″, *H*-4″, *H*-5″, *H*-6″), 7.27 (1H,

d, *H*-5′), 6.55 (1H, s, OC*H*OH), 4.27–4.25 (4H, s, -CH_2NCH_2), 3.97 (3H, s, OCH_3); ^{13}C-NMR (150 MHz, MeOD): 167.5, 160.3, 155.6, 134.3, 132.7, 132.3, 130.9, 130.6, 129.2, 128.6, 125.5, 124.9, 112.3, 119.5, 111.1, 107.4, 98.4, 55.5, 53.7; ESI-MS calcd. for $C_{19}H_{19}BrNO_4$: $[M + H]^+$ 374.0 and 376.0 (1:1); found 374.0 and 376.0 (1:1).

Acetic acid 3-benzo[b]thiophen-2-yl-5-oxo-2,5-dihydro-furan-2-yl ester (18)

Yield: 81%; ^{1}H-NMR δ (300 MHz, $CDCl_3$): 7.86 (1H, d, *H*-8′), 7.50 (1H, s, *H*-3′), 7.44 (3H, m, *H*-5′, *H*-6′, *H*-7′), 6.35 (1H, s, OC*H*OC-OCH_3), 2.23 (3H, s, CH_3); ^{13}C-NMR δ (75 MHz, $CDCl_3$): 170.5, 170.1, 157.1, 141.1 108.5, 139.3, 132.8, 128.6, 127.7, 125.4, 125.2, 122.3, 115.2, 22.1; HRMS calcd. for $C_{14}H_9O_4S$: $[M - H]^-$ 273.3, found 273.3.

Assay of sPLA$_2$

*s*PLA$_2$ activity was assayed using [^{3}H]oleate-labeled membranes of *E. coli*, following a modification of the method of Franson *et al.* (29,30). *E. coli* strain CECT 101 was grown for 6–8 h at 37 /C in the presence of 5 μCi/mL [^{3}H]oleic acid (specific activity 10 Ci/mmol) until the end of the logarithmic phase. After centrifugation at 1800 *g* for 10 min at 4 /C, the membranes were washed, resuspended in phosphate-buffered saline (PBS), and autoclaved for 30–45 min. At least 95% of the radioactivity was incorporated into the phospholipid fraction. Human recombinant synovial (group IIA *s*PLA$_2$) enzyme was used as source of *s*PLA$_2$. Enzyme was diluted in 10 μL of 100 mM Tris–HCl, 1 mM $CaCl_2$ buffer, pH 7.5, and preincubated at 37 /C for 5 min with 2.5 μL of test compound dissolved in ethanol or 2.5 μL of ethanol (control group) to get a final volume of 250 μL. Incubation proceeded for 15 min in the presence of 20 μL of [^{3}H]oleic-*E. coli* membranes and was terminated by addition of 100 μL ice-cold solution of 0.25% bovine serum albumin (BSA) solution in saline to a final concentration of 0.07% (w/v). After centrifugation at 2500 *g* for 10 min at 4 /C, the radioactivity (cpm) in the supernatants was determined by liquid scintillation counting.

Western blot assay of COX-2 and mPGES-1

Cellular lysates from RAW 264.7 (murine macrophages, 1.5×10^6 cells/mL) incubated for 18 h with LPS (1 μg/mL) were obtained with lysis buffer A [10 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES), pH 8.0, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*- tetraacetic acid (EGTA), 10 mM KCl, 1 mM dithiothreitol, 5 mM NaF, 1 mM Na_3VO_4, 10 mM Na_2MoO_4, 1 μg/mL leupeptin, 0.1 μg/mL aprotinin and 0.5 mM phenylmethanesulfonyl fluoride]. Following centrifugation (10 000 *g*, 15 min, 4 °C), supernatant protein was determined by the Bradford method with BSA as standard. COX-2 or *m*PGES-1 protein expression was studied in the total fraction or microsomal fractions, respectively. Equal amounts of protein (50 μg for both COX-2 and *m*PGES-1) were loaded on SDS–15% PAGE and transferred onto poly(vinylidene difluoride) membranes for 90 min at 125 mA. Membranes were blocked in PBS (0.02M pH 7.0)-Tween 20 (0.1%), containing 3% (w/v) nonfat milk and incubated with specific polyclonal antibody against COX-2 (1/1000) or *m*PGES-1 (1/200). Finally, membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1/10,000). The immunoreactive bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences).

Culture of murine macrophage RAW 264.7 cell line

The mouse macrophage cell line RAW 264.7 (Cell Collection, Department of Animal Cell Culture, C.S.I.C., Madrid, Spain) was cultured in Dulbecco's modified Eagle's medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% fetal bovine serum. Cultures were maintained at 37 °C in 5% CO_2 (air:CO_2 95:5) humidified incubator. Cells were resuspended at a concentration of 1.5×10^6 cells/mL.

PGE$_2$ production in RAW 264.7 macrophages

RAW 264.7 macrophages (1.5×10^6 cells/mL) were co-incubated in 96-well culture plate (200 μL) with 1 μg/mL of *E. coli* [serotype 0111:B4] lipopolysaccharide (LPS) at 37 °C for 20 h in the presence of 2.0 μL of test compound dissolved in ethanol or 2.0 μL of ethanol (control group). PGE$_2$ levels were determined in culture supernatants by radioimmunoassay (31). The mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (32) was used to assess the possible cytotoxic effects of compounds.

Statistical analysis

The results are presented as means ± SEM; *n* represents the number of experiments. Inhibitory concentration 50% (IC_{50}) values were calculated from at least four significant concentrations ($n = 6$). The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. Significance was assumed at a p value of 0.05 or less.

Results and Discussion

Chemistry

Considering that compound **2**, our target molecule, consists of a 3-bromo 4-substituted hydroxybutenolide, we decided to start with the substitution of the benzothiophene appendage with bioisosteric moieties, such as indole and benzofurane units **6a** and **6b** (see chemistry section); as a result, we observed a clear loss of activity, especially for benzofurane derivative (Table 1). Then, we took into consideration another small array of 4-differently substituted 3-bromo-hydroxybutenolides, **6c-f**. Among these, compounds **6e** and **6f** belong to a small collection of products generated by *Ludi* (12–14) in the course of our previous medicinal chemistry project focused on the discovery of potential PLA$_2$ inhibitors. On the contrary, **6c** and **6d** were conceived to mimic the same structural moiety present in resveratrol, which has recently emerged as a potent modulator of *m*PGES-1 expression (15). Unfortunately, we did not observe an activity increase with respect to the lead compound **2** for any of the previous molecules. Afterward, we decided to investigate the role played by the bromine atom, and for this purpose, we

Table 1: Inhibitory activity and cytotoxic effect of the γ-hydroxybutenolide derivatives at 10 μM on the production of PGE_2 in LPS-stimulated RAW 264.7 cells.

Compound 10 μM	% Inhibition	IC_{50} (μM)	% Toxicity
6a	81.6 ± 4.7**	4.20	<5.0
6b	46.7 ± 6.1**	n.d.	<5.0
6c	45.1 ± 9.7**	n.d.	<5.0
6d	46.5 ± 11.1**	n.d.	<5.0
6e	61.7 ± 8.1**	7.30	<5.0
6f	72.2 ± 9.6**	4.80	<5.0
14g	100.0 ± 0.0**	0.85	28.9 ± 3.0**
15g	85.1 ± 3.5**	3.40	<5.0
16g	87.1 ± 3.9**	1.25	<5.0
16h	<20.0	n.d.	<5.0
16i	76.0 ± 5.7**	3.17	<5.0
16j	<20.0	n.d.	<5.0
16k	<20.0	n.d.	<5.0
17g	65.9 ± 8.1**	4.46	<5.0
17h	<20.0	n.d.	<5.0
17i	n.d.	n.d.	89.9 ± 0.2**
17j	<20.0	n.d.	<5.0
17k	<20.0	n.d.	<5.0
18	100.0 ± 0.0**	0.79	<5.0
2	72.2 ± 5.7**	1.80	<5.0

Results show means ± SEM (n = 6). Statistical significances: **p < 0.01, with respect to the LPS-stimulated control group. PGE_2 (non-stimulated cells = 0.6 ± 0.2 ng/mL; LPS-stimulated cells = 16.0 ± 1.6 ng/mL). n.d. = not determined.

examined the effects of two arrays of regioisomeric debrominated γ-hydroxybutenolides **16g-k** and **17g-k,** respectively, previously synthesized by us, in the frame of a project focused on a synthetic implementation task and re-synthesized, for the present purpose, using the same strategy reported by us, the photooxidation of 3-bromofuran with singlet oxygen in the presence of a suitable base (16). In the case of **16g**, the debrominated **2**, we observed a moderate activity increase. On the contrary, the other 4-substituted butenolides **16h-k** (except **16i**, which showed a discrete potency) were found completely inactive. The only consideration emerged actually from these preliminary results is that the only structural change proved to be effective for the activity was the elimination of bromine atom from the C-3 position of the lead compound **2**. In line with these findings, the other array of regioisomeric compounds **17g-k,** all presenting substitutions on C-3 position of the scaffold, did not give satisfactory results. Finally, we decided to perform on compound **16g,** the most active one so far obtained, the protection of OH either with acetyl **18** or with THP group **14g** to verify whether the masked aldehyde was crucial for the activity, as it was proved to be for the inhibition mechanism of the other known natural butenolides (2,17,18). Compounds **18** and **14g** at last displayed a higher potency in inhibiting the expression of *m*PGES-1, in comparison with the reference structure **2**, which is of relevance in consideration that only very few compounds are known to affect the level of *m*PGES-1 enzyme, so that the availability of pharmacologically active molecules capable of selective modulate *m*PGES-1 expression may provide new tools for better therapeutic approach.

For the synthesis of compounds **6a-f**, according to Scheme 1, we first protected the mucobromic acid **3** with methoxy-ethoxy-methylchloride (MEM-Cl) (19) and then we connected the MEM-protected butenolide **4** with the boronic acids $R\text{-}B(OH)_2$ or their respective pinacolic esters (20), using the Suzuki coupling reaction (21), previously optimized by us (22). To speed up the reaction, we took advantages of microwaves heating strategy (23), which gave compounds **5a-f** in good yields, together with some by-products, mainly consisting of bis-coupling and homo-coupling adducts. Finally, the removal of MEM protecting group afforded the desired compounds **6a-f**.

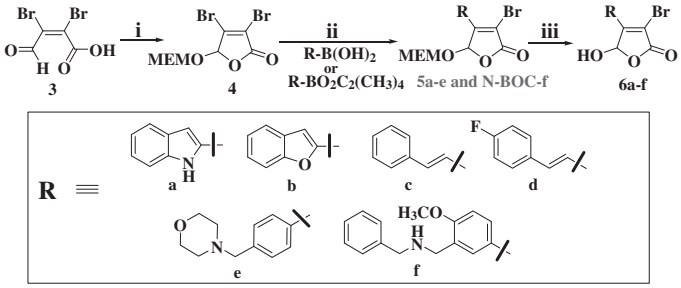
All the boronic partners utilized for the synthesis of **6a-f** are commercially available except for **e** and **N-BOC-f**. These last were obtained, according to Scheme 2, through a convergent synthetic approach. Based on this, we first assembled the molecular appendages by a reductive amination between the pinacolic esters **9** and **10** with the amines **11** and **12**, respectively, and then we performed the Suzuki coupling to connect these fragments to the butenolide scaffold **4**.

The two regioisomeric arrays of compounds **16g-k** and **17g-k** (Figure 2), respectively, were obtained utilizing the optimized protocol of photooxidation reaction performed on 3-bromofuran in the presence of a suitable base, as previously reported by us (16).

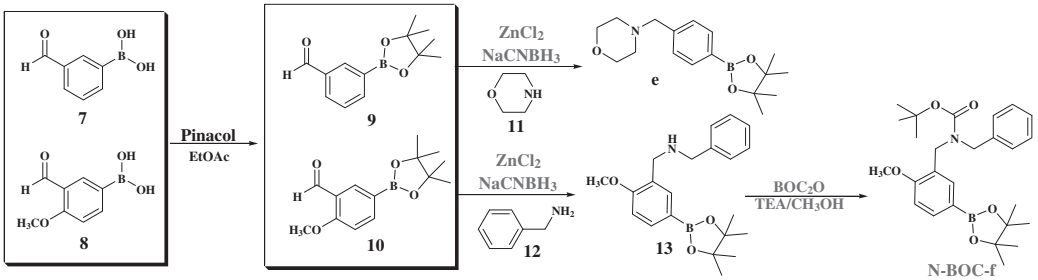
Finally, the acetylation of compound **16g** furnished the desired compound **18** (Scheme 3).

Biology

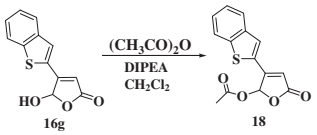
Enzymatic inhibition of $sPLA_2$, mainly type IIA $sPLA_2$, is a pharmacological approach that can modulate the availability of arachidonic



Scheme 1: Synthesis of compounds **6a-f**. [a]Reagents and conditions: (i) MEM-Cl, DIPEA, CH_2Cl_2, rt, 4h; (ii) $Pd(dppf)_2Cl_2$, TBAB, CsF, THF/H_2O 1:1, MW, 120°C, 3-6 min; (iii) TFA (95%), TIS (2.5%), H_2O (2.5%).



Scheme 2: Synthesis of appendages **e** and **N-BOC-f**.



Scheme 3: Acetylation of compound **16g**.

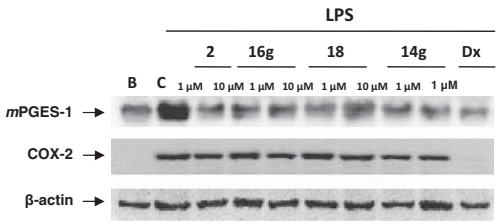


Figure 3: Effect of γ-hydroxybutenolide derivatives on *m*PGES-1 and COX-2 expression in LPS-stimulated RAW 264.7 cells. The figure is representative of two similar experiments. B: normal cells. C: LPS-stimulated cells. Dx. dexamethasone.

acid and consequently the production of PGE_2 and the inflammatory process (24). Among the nineteen new γ-hydroxybutenolide derivatives tested at 10 μM, under the same experimental conditions, on $sPLA_2$ belonging to the group IIA (human synovial recombinant), only compound **6e** exerted a weak inhibitory profile (30.4%). All the other γ-hydroxybutenolide derivatives tested were devoid of significant inhibition (data not shown) against this pro-inflammatory enzyme, unlike did the reference inhibitor LY311727 (96.3%) used as a reference tool (25).

We determined the effect of the nineteen γ-hydroxybutenolide derivatives on PGE_2 production on mouse macrophage cell line RAW264.7 stimulated with LPS (Table 1).

After 18-h stimulation, compounds **2**, **6a**, **6e**, **6f**, **14g**, **15g**, **16g**, **16i**, **17g**, and **18** were able to inhibit PGE_2 production with a percentage of inhibition higher than 50% at 10 μM, showing IC_{50} values in the micromolar range. Only compounds **18, 14g**, and **16g** exerted an inhibitory potency higher than the leader compound **2**. This profile is especially relevant for compounds **18** and **14g**, the two protected derivatives of the debrominated **2.** On the other hand, all the derivatives except **17i,** which was discarded, were devoid of significant cytotoxic effects on RAW264.7 at concentrations up to 10 μM, as assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (Table 1). Compound **14g** showed a slight cytotoxic effect that disappeared at lower concentrations.

Western blot analysis for *m*PGES-1 and COX-2 proteins using 18-h LPS-stimulated RAW 264.7 cells (Figure 3) shows clearly that compounds **18, 14g**, and **16g** and the leader compound **2** inhibit *m*PGES-1 expression, without any effect on COX-2 expression, whereas dexamethasone, as expected, reduced the expression of both inducible proteins.

COX-2 and *m*PGES-1 are the main inducible enzymes responsible for PGE_2 synthesis (26), and both of them are downregulated by glucocorticoids in various cells (27,28). The use of Non Steroidal Anti-Inflammatory Drugs (NSADs) and glucocorticoids is a mainstay of anti-inflammatory therapy. Inhibition of PGE_2 formation by COX-2 inhibitors is effective in ameliorating symptoms of inflammation. However, the cardiovascular side effects associated with COX-2 inhibitors have limited their use. The concomitant increased cardiovascular safety observed in *m*PGES-1-deficient animals compared with COX-2 inhibition under similar conditions makes *m*PGES-1 an attractive target for development of a new class of therapeutic agents. The selective pharmacological profile exerted by these compounds could be of great interest to discover new promising drugs, as well as to provide pharmacological tools to discern the role of *m*PGES-1 and COX-2 in a great variety of inflammatory disorders.

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Artículo 6

Perthamides C–F, potent human antipsoriatic cyclopeptides

Festa C, De Marino S, Sepe V, D'Auria MV, Bifulco G, Andrés R, Terencio MC, Payá M, Debitus C, Zampella A

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Perthamides C–F, potent human antipsoriatic cyclopeptides

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ABSTRACT

Two new cyclopeptides, perthamides E and F were isolated from the polar extracts of the sponge *Theonella swinhoei*. The new structures, featuring an unprecedented β-amino acid unit (AHMOA), were determined by interpretation of NMR and MS data. The absolute configuration of the AHMOA residue was proposed on the basis of quantum chemical calculation of NMR chemical shifts. Perthamides were proved to inhibit TNF-α and IL-8 release in primary human keratinocytes cells and therefore could represent potentially leads for the treatment of psoriasis.

1. Introduction

Psoriasis is a chronic autoimmune inflammatory skin disorder affecting approximately 2–3% of the general population in Europe and North America. Cutaneous and systemic overexpression of various proinflammatory cytokines (TNF-α, IL-8, IFN-γ, etc.) has been demonstrated in psoriatic patients.[1,2] Notably it has been postulated that TNF-α produced locally in psoriatic lesions creates a TNF-α positive feedback loop that amplifies and sustains the inflammatory process within plaques.[3] In fact, recently developed anti-inflammatory therapies based on blocking TNF-α signalling have been shown to be effective in the treatment of psoriasis and could become a highly promising option for the treatment of this skin condition.[4,5]

Recently, we reported the isolation and the chemical characterization of two cyclic octapeptides, which we named perthamides C (**1**) and D (**2**), from the Solomon marine sponge *Theonella swinhoei*.[6,7] Perthamide C has an unprecedented primary structure that comprises a 25-membered macrocycle with 6 out the 8 residues constituted by unusual amino acids: γ-methylproline, N^{δ}-carbamoyl-β-OSO_3asparagine, *o*-tyrosine, D-Abu, *O*-methylthreonine and the β-amino acid AHMHA (3-amino-2-hydroxy-6-methylheptanoic acid). Perthamide C (**1**), when tested in a well characterised model of inflammation in vivo, i.e., mouse paw oedema, significantly reduced carrageenan-induced paw oedema, displaying a dose-dependent anti-inflammatory activity.

Despite this promising activity, the mechanism of action at the molecular level of perthamide C is unknown. Although the modular peptidic nature and the ready chemical access to perthamide C may open the possibility to investigate new pharmacophores, the isolation of further natural derivatives represents an alternative approach to investigate the structure–activity relationships and to shed light on the biological target of this promise anti-inflammatory lead. In this respect, the sponge *T. swinhoei* (order Lithistida, family Theonellidae), recognized as one of the most prolific sources of bioactive secondary metabolites,[8] could represent a source of useful perthamide derivatives. Continuing investigation of the polar extracts of this sponge afforded a large amount of perthamide C together with two new derivatives, perthamides E (**3**) and F (**4**).

In this paper we describe the isolation, the structure elucidation including the stereochemical characterization and the biological activity of the new peptides (Fig. 1).

2. Results and discussion

The lyophilized sponge (400 g) was extracted with MeOH, and the combined extracts were fractionated according to the Kupchan

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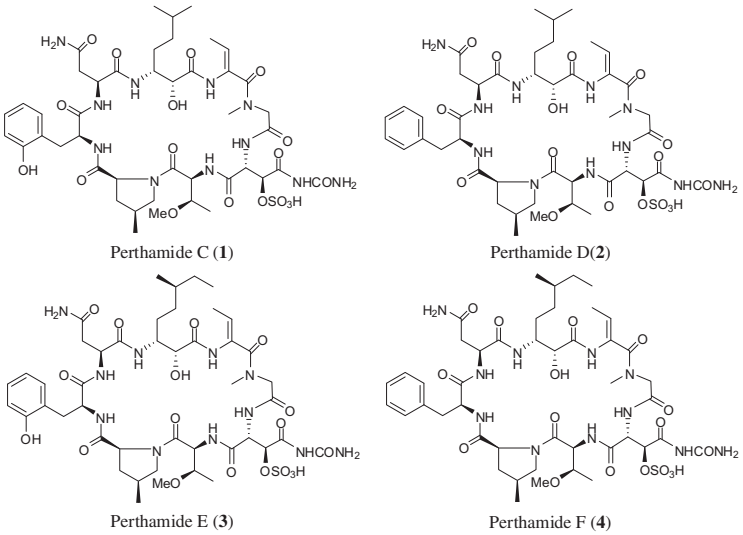

Fig. 1.

partitioning procedure.[9] The *n*-BuOH extract was purified by DCCC (*n*-BuOH/Me_2CO/H_2O, descending mode) followed by reverse-phase HPLC to afford perthamides C–F.

Perthamide E (**3**) was isolated as a colourless amorphous solid and showed the pseudomolecular ion peak at *m*/*z* 1080.4361 $[M-H]^-$ in the HRESIMS spectrum, corresponding to the molecular formula $C_{45}H_{67}N_{11}O_{18}S$ and indicating the presence of one additional carbon atom with respect to perthamide C (**1**).

A careful analysis of the NMR data (Table 1), including COSY, HSQC, TOCSY, indicated the presence of the same α-amino acid residues found in **1** and a variation of the β-amino acid unit (AHMHA in perthamide C). Even if from ^{1}H NMR and HSQC data an additional methylene group with respect to perthamide C was easily detected (δ_C 28.7, δ_H 1.03, 1.22), the analysis of the proton spin system of this amino acid unit was not straightforward due to the heavy overlap in the ^{1}H NMR high field region and to the absence of some scalar coupling in the COSY spectrum. The ^{1}H and ^{13}C NMR resonances of C-1/C-5 nuclei relative to this portion were almost superimposable with the corresponding values found for the 3-amino-2-hydroxy-6-methylheptanoic acid (AHMHA) residue in perthamide C. Additionally, inspection of ^{1}H NMR spectrum indicated the presence of one methyl doublet (0.74, d, *J*=6.2 Hz) and

Table 1
NMR data (700 MHz, DMSO-d_6) of perthamides E (**3**) and F (**4**)

Position	Perthamide E (**3**)		Position	Perthamide F (**4**)	
	δ_H (*J* in Hz)[a]	δ_C[a]		δ_H (*J* in Hz)[a]	δ_C[a]
ThrOMe			*ThrOMe*		
1	—	170.9	1	—	170.9
2	4.91d (9.5)	55.4	2	4.81d (9.6)	55.4
3	4.16m	72.9	3	4.14–4.22m	72.9
4	1.23d (5.9)	14.3	4	1.21d (5.8)	14.4
OMe	3.26s	54.7	OMe	3.25s	54.5
NH	9.01d (9.1)		NH	8.90d (8.8)	
γMePro			*γMePro*		
1	—	170.7	1	—	170.7
2	3.89dd (11.1, 6.6)	63.4	2	3.98dd (11.0, 6.7)	63.3
3	0.71m, 2.05–2.13m	36.2	3	1.09 ovl, 2.14–2.18m	36.3
4	2.20–2.30m	33.2	4	2.19–2.27m	33.5
5	3.39 ovl, 4.11 ovl	53.2	5	3.38 ovl, 4.10 ovl	53.2
6	1.03d (5.8)	15.8	6	1.01d (5.7)	15.6
o-Tyr			*Phe*		
1	—	170.6	1	—	170.6
2	4.11 ovl	56.4	2	4.28–4.36m	55.8
3	2.86dd (13.2, 3.2), 2.93t (13.2)	30.7	3	2.83dd (13.5, 3.5), 3.14 ovl	36.2
1′	—	124.3	1′	—	137.8
2′	—	154.6	2′	7.19d (7.4)	128.0
3′	6.87d (7.8)	114.5	3′	7.30t (7.4)	128.3
4′	7.10t (7.8)	128.0	4′	7.22d (7.3)	126.3
5′	6.80t (7.3)	119.9	5′	7.30t (7.4)	128.3
6′	7.16 ovl	130.5	6′	7.19d (7.4)	128.0
NH	7.20 ovl		NH	6.91d (8.0)	

(*continued on next page*)

Table 1 (*continued*)

Position	Perthamide E (**3**)		Position	Perthamide F (**4**)	
	δ_H (*J* in Hz)[a]	δ_C[a]		δ_H (*J* in Hz)[a]	δ_C[a]
OH	10.1s		OH	—	
Asn			*Asn*		
1	—	170.4	1	—	170.4
2	4.67–4.71m	48.0	2	4.66 br s	47.9
3	2.41d (16.3), 3.14dd (16.3, 4.7)	36.8	3	2.43d (16.0), 3.15 ovl	36.8
4	—	172.6	4	—	172.5
NH	7.19 ovl		NH	7.12 ovl	
NH_2	6.52 br s, 7.83 br s		NH_2	6.55 br s, 7.80 br s	
AHMOA			*AHMOA*		
1	—	169.8	1	—	169.8
2	4.14 ovl	72.8	2	4.12 ovl	72.9
3	4.01t (9.9)	51.9	3	4.01t (9.8)	52.0
4	1.09m, 1.41–1.47m	23.6	4	1.07 ovl, 1.36–148m	23.5
5	1.06m	31.8	5	1.04m	31.6
6	1.17m	33.4	6	1.19m	33.3
7	1.03m, 1.22 ovl	28.7	7	1.05m, 1.23m	28.8
8	0.76t (7.0)	11.0	8	0.77t (6.9)	11.1
9	0.74d (6.2)	18.5	9	0.75d (6.2)	18.4
NH	6.37d (9.1)		NH	6.43d (9.0)	
OH	4.81 br s		OH	4.81 br s	
d-Abu			*d-Abu*		
1	—	167.5	1	—	167.4
2	—	132.3	2	—	132.1
3	5.77q (6.8)	126.5	3	5.75q (6.5)	126.0
4	1.54d (6.8)	12.0	4	1.54d (6.5)	12.0
NH	8.62s		NH	8.66s	
NMeGly			*NMeGly*		
1	—	167.2	1	—	167.2
2	3.31d (17.5), 4.19d (17.5)	51.5	2	3.30 ovl, 4.18d (17.0)	51.6
NMe	2.70s	34.6	NMe	2.70s	34.6
N^{δ}-c-β-OSO_3Asn			*N^{δ}-c-β-OSO_3Asn*		
1	—	167.5	1	—	167.2
2	5.04t (7.3)	53.0	2	5.01t (7.3)	52.9
3	4.59d (7.1)	74.9	3	4.58d (6.9)	75.0
4	—	170.5	4	—	170.5
βNH	7.16 ovl		βNH	7.15 ovl	
NH	8.89s		NH	8.93s	
CO	—	n.o	CO	—	n.o
NH_2	7.29 br s, 7.51 br s		NH_2	7.33 br s, 7.50 br s	

Ovl: overlapped; n.o: not observed.
[a] ^1H and ^{13}C assignments aided by COSY, TOCSY, HSQC and HMBC experiments.

one methyl triplet (0.76, t, *J*=7.0 Hz) that replaced the two highly overlapped methyl doublet signals at δ_H 0.77 and 0.78 observed in the ^1H NMR spectrum of **1**, suggesting the presence of a terminal appendage for this unit different from the isopropyl group located on AHMHA.

Two spin systems, a CH—CH_3 (δ_H 1.17, δ_C 33.4, C-6; δ_H 0.74, δ_C 18.5, C-9) and a CH_2—CH_3 (δ_H 1.03, 1.22, δ_C 28.7, C-7; δ_H 0.76, δ_C 11.0, C-8) were disclosed from COSY and HSQC data (Fig. 2). Even if no COSY correlation was observed between the proton at δ_H 1.17 (H-6) and protons ascribable to the methylene at C-7, the connection between C-6 and C-7 was inferred by HMBC data as shown in Fig. 2, defining a *sec*-butyl subunit. The linkage of this latter with the methylene carbon at C-5 (δ_C 31.8, δ_H 1.06) was deduced from the diagnostic heteronuclear long range correlation between the methyl protons at δ_H 0.74 (C-9) with the carbon resonance at δ_C 31.8 (C-5). Definitive confirmation to the above unit derived from 2D-TOCSY analysis that showed correlations starting from H-9 to H-3.

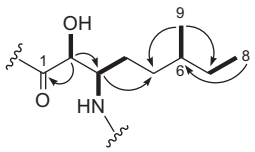

Fig. 2. AHMOA unit in perthamides E and F with COSY/TOCSY connectivities (bold bonds) and HMBC correlations (arrows).

Therefore the new 3-amino-2-hydroxy-6-methyloctanoic acid (AHMOA) unit, which is also unprecedented in natural peptides, was established in perthamide E.

As shown in Fig. 3, the sequence of the amino acids in perthamide E (**3**) was established on the basis of careful analysis of ROESY data and was proved to be the same found in **1**.

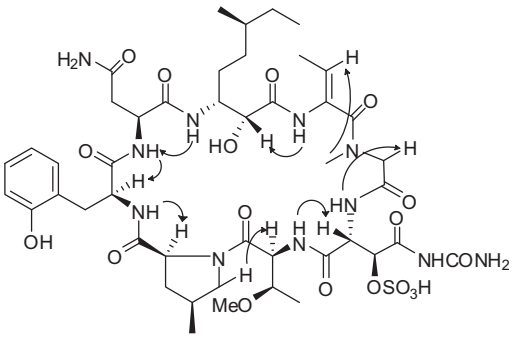

Fig. 3. Selected ROE correlations for perthamide E.

The close resemblance of ^1H and ^{13}C NMR chemical shifts of all α-amino acid residues with the corresponding values of perthamide C suggested that they have the same configuration. To confirm

this hypothesis, **3** was subjected to the same procedures described for the stereochemical characterization of the amino acid residues in perthamide C,[6,7] establishing the presence of L-Asn, L-*o*-Tyr, *cis*-L-γMePro, L-ThrOMe, *erythro*-D-N^{δ}-C-β-OSO_3Asn and Z-D-Abu.

As concern AHMOA, this unit has an additional stereogenic centre with respect to AHMHA in perthamide C. Chemical shifts and the homonuclear coupling constant pattern of C-2/C-3 centres closely matched with those of the corresponding centres in AHMHA for which the 2*R*,3*R* absolute configuration was established by stereoselective synthesis.[7] The impossibility to relate this stereotriad to C-6 through the intervening of two methylene carbon that showed highly overlapped proton resonances, hampered the application of *J*-based analysis to define the stereochemistry at C-6.

Starting from the encouraging results recently obtained from our research group in the solving of stereochemical ambiguities on marine natural products from *T. swinhoei*,[10,11] we decided to apply quantum chemical (QM) calculation of the NMR chemical shifts[12,13] to establish the absolute configuration of AHMOA residue in **3**.

Two diastereoisomers of perthamide E (**3a** and **3b**, Fig. 4) were built, varying the configuration at C-6 of AHMOA residue and fixing the absolute configuration of all residues previous established by Marfey's analysis with perthamide C as standard.

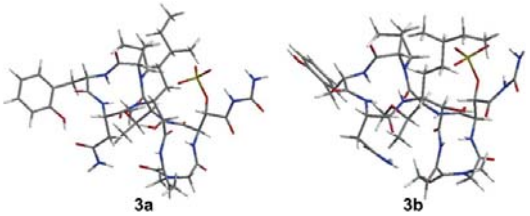

Fig. 5. NMR solution state representative conformations for **3a** and **3b** obtained by restrained MD calculations, using ROESY data collected at 700 MHz in DMSO-d_6.

the two diastereoisomers. Through evaluation of the MAE values, the GIAO calculated ^{13}C and ^{1}H NMR chemical shifts of nuclei of AHMOA residue indicated the best fit between stereoisomer 2*R*,3*R*,6*S* (**3b**) and the experimental data.

Even though small differences in the MAE values of calculated ^{13}C chemical shifts were observed (4.2 for **3a** vs 3.9 for **3b**, Table 2), MAE values of calculated ^{1}H chemical shifts were relatively enough different (0.40 for **3a** vs 0.24 for **3b**, Table 3) to propose the 2*R*,3*R*,6*S* absolute configuration for AHMOA residue. Definitive confirmation

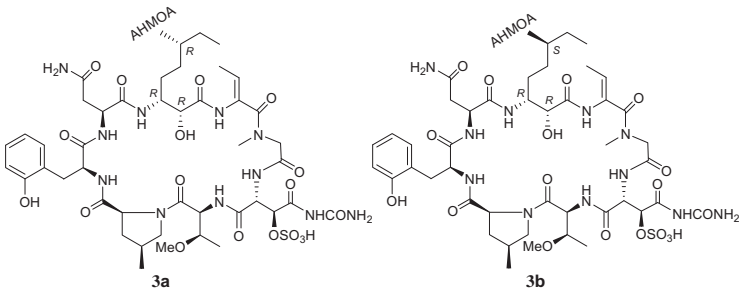

Fig. 4. AHMOA-C6 epimers (**3a**,**b**) of perthamide E (**3**).

The adopted protocol consists of four fundamental steps: (a) conformational search and preliminary geometry optimization of all the significantly populated conformers of each stereoisomer; (b) geometry optimization of all the species at MPW1PW91 level; (c) GIAO ^{13}C and ^{1}H NMR calculations of all the so obtained structures; (d) comparison of the Boltzmann averaged ^{13}C and ^{1}H NMR chemical shifts calculated for each stereoisomer with those measured for perthamide E.

The preliminary conformational search was performed by empirical force field molecular dynamics (OPLS force field, Macromodel 8.5 software).[14] A set of distance restraints were collected from 2D-ROESY NMR experiments (t_{mix}=150 ms) and used in molecular dynamics calculations (500 K) of two diastereoisomers.

Subsequently, all the conformers found were optimised at DFT level by using the MPW1PW91 functional and 6-31G(d) basis set. By discarding all of the conformations higher in energy than a threshold of 10 kcal/mol from the most stable species, five major conformers were found for stereoisomer **3a** and five for **3b**. These showed a well defined macrocycle core, but differed in the conformational structure of the side chain of AHMOA residue (Fig. 5).

Finally, GIAO ^{13}C and ^{1}H chemical shift calculations were performed at the MPW1PW91 functional and 6-31G (d,p) basis set on each set of conformers relative to the epimers **3a**,**b**, and calculated by simulating the presence of DMSO (IEF-PCM),[15] using Gaussian 09 software.[16] For each stereoisomer, the ^{13}C and ^{1}H chemical shifts were obtained as the weighted average chemical shift values in all of the conformers sampled by the initial conformational search. MAE (Mean Absolute Error) parameters were obtained for each of to the above tentatively assigned absolute configuration could derive from synthetic studies already ongoing in our laboratories.

Table 2
Experimental and calculated ^{13}C NMR chemical shifts (ppm), ΔΔ [($\delta_{calcd}-\delta_{exptl}$)], ΔΔabs [|($\delta_{calcd}-\delta_{exptl}$)|] and MAE (Mean average error as $\sum|(\delta_{exptl}-\delta_{calcd}|)/n$) on selected sp^3 atoms of AHMOA residue in **3**

	2*R*,3*R*,6*R*				2*R*,3*R*,6*S*		
	δ_{exptl}	δ_{calcd}	ΔΔ	ΔΔabs	δ_{calcd}	ΔΔ	ΔΔabs
C2	72.8	78.7	−5.9	5.9	77.3	−4.5	4.5
C3	51.9	58.8	−6.9	6.9	53.2	−1.3	1.3
C4	23.6	31.6	−8.0	8.0	36.8	−13.2	13.2
C5	31.8	38.5	−6.7	6.7	31.6	0.2	0.2
C6	33.4	34.1	−0.7	0.7	36.5	−3.1	3.1
C7	28.7	30.7	−2.0	2.0	32.3	−3.6	3.6
C8	11.0	14.2	−3.2	3.2	14.0	−3.0	3.0
C9	18.5	18.7	−0.2	0.2	21.5	−3.0	3.0
$\sum[\delta_{exptl}-\delta_{calcd}]$			−33.6	33.6		−31.5	31.9
$\sum[\delta_{exptl}-\delta_{calcd}]/n$			−4.2			−3.9	
MAE[a]				4.2			3.9

[a] Mean average error=$\sum[|\delta_{exptl}-\delta_{calcd}|]/n$.

Perthamide F (**4**) showed a molecular weight 16 mass units (one oxygen atom) smaller than that of perthamide E (**3**). The ^{1}H NMR spectrum, which is very similar to that of **3**, clearly revealed the absence of the signal ascribable to exchangeable OH proton located on the *o*-Tyr residue in perthamides C and E and a perturbation in the aromatic region.

Table 3
Experimental and calculated ^{1}H NMR chemical shifts (ppm), ΔΔ [($\delta_{calcd}-\delta_{exptl}$)], ΔΔabs [|($\delta_{calcd}-\delta_{exptl}$)|] and MAE (Mean average error as $\sum[|\delta_{exptl}-\delta_{calcd}|]/n$) on selected sp^3 atoms of AHMOA residue in **3**

	2*R*,3*R*,6*R*				2*R*,3*R*,6*S*		
	δ_{exptl}	$\delta_{calc.}$	ΔΔ	ΔΔabs	$\delta_{calc.}$	ΔΔ	ΔΔabs
H2	4.14	4.18	−0.04	0.04	4.36	−0.22	0.22
H3	4.01	3.88	0.13	0.13	4.03	−0.02	0.02
H4a	1.09	1.96	−0.87	0.87	1.60	−0.51	0.51
H4b	1.44	2.26	−0.82	0.82	1.77	−0.33	0.33
H5	1.06	1.29	−0.23	0.23	1.54	−0.48	0.48
H6	1.17	1.94	−0.77	0.77	1.22	−0.05	0.05
H7a	1.03	0.92	0.11	0.11	1.40	−0.37	0.37
H7b	1.22	1.86	−0.64	0.64	1.10	0.12	0.12
H8	0.76	0.99	−0.23	0.23	0.91	−0.15	0.15
H9	0.74	0.92	−0.18	0.18	0.85	−0.11	0.11
$\sum[\delta_{exptl}-\delta_{calcd}]$			−3.5	4.0		−2.1	2.4
$\sum[\delta_{exptl}-\delta_{calcd}]/n$			−0.35			−0.21	
MAE[a]				0.40			0.24

[a] Mean average error=$\sum[|\delta_{exptl}-\delta_{calcd}|]/n$.

Interpretation of 2D NMR data, including COSY, HSQC and HMBC data (Table 1) indicated that perthamide F (**4**) possesses similar primary structure to that of perthamide E (**3**), with the differences traced to substitution of *o*-Tyr in **3** with Phe in **4**. As for in perthamide E, the presence of L-Asn, L-Phe, *cis*-L-γMePro, L-ThrOMe, *erythro*-D-N^{δ}-C-β-OSO_3Asn was established by application of Marfey's method,[17] whereas the complete agreement of chemical shifts and coupling constant pattern of AHMOA and D-Abu residues suggested the same configuration.

Starting from the anti-inflammatory activity demonstrated for perthamide C, we decided to investigate the eventual antipsoriatic effect of these natural compounds on TNF-α and IL-8 release in primary human keratinocytes (PHK) cells (Fig. 6).

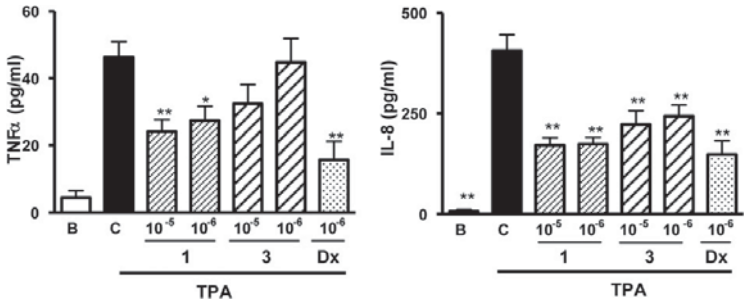

Fig. 6. Dose-dependent inhibition of TNF-α and IL-8 production by perthamide C (**1**) and E (**3**) in PHK cells. B: non stimulated cells. C: stimulated cells. Dx: Dexamethasone.

Dexamethasone, a well known antipsoriatic drug, was used as a reference tool. Perthamides C and E were devoid of significant cytotoxic effects on PHK cells at concentrations up to 10 μM, as assessed by the mitochondrial-dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan (data not shown). Their ability to inhibit TNF-α and IL-8 release on PHK cells stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was also investigated (Fig. 6). Interestingly, perthamide C (**1**) showed a dose-dependent capability to inhibit TNF-α and IL-8 release, whereas perthamide E (**3**) significantly inhibited only IL-8 release. The ability of perthamide C to inhibit both key biomarkers upregulated in the inflammatory response of psoriatic skin, could be interesting to provide promising antipsoriatic agents.

In conclusion in this paper we report the isolation and the stereostructural characterization of two new cyclic peptides from the marine sponge *T. swinhoei*.

Moreover we demonstrated that perthamides C and E are endowed with anti-inflammatory activity mediated via up-regulation of TNF-α, a key cytokine in inflammatory skin diseases, such as psoriasis.

3. Experimental section

3.1. General experimental procedures

Specific rotations were measured on a Jasco P-2000 polarimeter. The melting points were measured on a Büchi Melting Point B-440 apparatus. IR spectra were recorded on a Perkin–Elmer FT-IR 100 spectrometer. High-resolution ESIMS spectra were performed with a Micromass QTOF spectrometer. ESIMS experiments were performed on an Applied Biosystem API 2000 triple-quadrupole mass spectrometer. NMR spectra were obtained on Varian Inova 500 and Varian Inova 700 NMR spectrometers (^{1}H at 500 and 700 MHz, ^{13}C at 125 and 175 MHz, respectively) equipped with a Sun hardware, δ (ppm), *J* in Hz, spectra referred to DMSO-d_6 as internal standard (δ_H 2.50, δ_C 39.5). HPLC was performed using a Waters 510 pump equipped with Waters Rheodine injector and a differential refractometer, model 401.

Through-space ^{1}H connectivities were evidenced using a ROESY experiment with mixing time of 150 ms.

DCCC was performed using a DCC-A (Rikakikai Co. Di Tokyo) equipped with 250 columns (internal diameter 3 mm).

The purities of compounds were determined to be greater than 95% by HPLC, MS and NMR.

3.2. Sponge material and separation of individual peptides

T. swinhoei (order Lithistida, family Theonellidae) was collected at a depth of 22 m, on an isolated reef off the western coast of Malaita Island, Solomon Islands, in July 2004. The samples were frozen immediately after collection and lyophilized to yield 400 g of dry mass. Taxonomic identification was performed by Dr John Hooper at Queensland Museum, Brisbane, Australia, where a voucher specimen is deposited under the accessing number G322662.

The lyophilized material (400 g) was extracted with methanol (3×1.5 L) at room temperature and the crude methanolic extract was subjected to a modified Kupchan's partitioning procedure as follows. The methanol extract was dissolved in a mixture of MeOH/H_2O containing 10% H_2O and partitioned against *n*-hexane (15.2 g). The water content (% v/v) of the MeOH extract was adjusted to 30% and partitioned against $CHCl_3$ (5.83 g). The aqueous phase was concentrated to remove MeOH and then extracted with *n*-BuOH (6.0 g).

The *n*-BuOH extract (6.0 g) was chromatographed in three runs by DCCC using *n*-BuOH/Me_2CO/H_2O (3:1:5) in the descending mode

(the upper phase was the stationary phase), flow rate 8 mL/min; 4 mL fractions were collected and combined on the basis of their similar TLC retention factors.

Fractions 6–12 (1.1 g) were purified in many runs by HPLC on a reverse phase C-12 Jupiter Proteo column (Phenomenex, 4 μ, 250×4.6 mm, 1.0 mL/min), eluting in isocratic mode with 59% MeOH/H_2O (0.1% TFA) to afford 265.3 mg of perthamide C (t_R=3.5 min) and 10.5 mg of perthamide E (t_R=6.9 min).

Fractions 13–14 (113 mg) were purified by HPLC on a reverse phase C-12 Jupiter Proteo column (Phenomenex, 4 μ, 250×4.6 mm, 1.0 mL/min), with 59% MeOH/H_2O (0.1% TFA). The peak at t_R=6.9 min was analyzed by ^{1}H NMR and revealed a mixture of perthamides E and F. This mixture was purified by HPLC on Vydac C-18 column (5 μ, 250×4.6 mm, 1.0 mL/min) with 24% MeCN/H_2O as eluent to give 2.3 mg of perthamide E (t_R=6.3 min) and 1.7 mg of perthamide F (t_R=8.4 min).

3.3. Characteristic data for each peptide

*3.3.1. Perthamide C (**1**).* White amorphous solid; $[\alpha]_D^{22}$ −87.7 (*c* 0.87, MeOH); IR ν_{max} (KBr disc)/cm^{-1} 3395, 2932, 1665, 1618, 1522, 1457, 1234, 1130, 1083, 1028; mp 210.8–214.6 °C; HRESIMS *m*/*z* 1066.4106 [M−H]$^-$ (calcd for $C_{44}H_{64}N_{11}O_{18}S$: 1066.4151).

*3.3.2. Perthamide E (**3**).* White amorphous solid; $[\alpha]_D^{22}$ −199.6 (*c* 0.22, MeOH); IR ν_{max} (KBr disc)/cm^{-1} 3395, 2950, 1670, 1618, 1533, 1457, 1251, 1130, 1080, 1031; mp 204.1–211.6 °C; ^{1}H and ^{13}C NMR data in DMSO-d_6 given in Table 1; HRESIMS *m*/*z* 1080.4361 [M−H]$^-$ (calcd for $C_{45}H_{66}N_{11}O_{18}S$ *m*/*z* 1080.4308).

*3.3.3. Perthamide F (**4**).* White amorphous solid; $[\alpha]_D^{22}$ −10.5 (*c* 0.07, MeOH); ^{1}H NMR and ^{13}C NMR data in DMSO-d_6 given in Table 1; HRESIMS *m*/*z* 1064.4313 [M−H]$^-$ (calcd for $C_{45}H_{66}N_{11}O_{17}S$ *m*/*z* 1064.4359).

3.4. Determination of amino acid absolute configuration

3.4.1. Hydrolysis of perthamides. Perthamides (500 μg) C–F were dissolved, respectively, in 0.5 mL of 6 N HCl in three evacuated glass tubes and heated at 160 °C for 16 h. The solvent was removed in vacuo and the resulting material was subjected to further derivatisation.

3.4.2. LC–MS analysis of Marfey's (FDAA) derivatives. A portion of the hydrolysate mixture (300 μg) was dissolved in 80 μL of a 2:3 solution of TEA/MeCN and this solution was then treated with 75 μL of 1% 1-fluoro-2,4-dinitrophenyl-5-L-alaninamide (L-FDAA) in 1:2 MeCN/Me_2CO. The vials were heated at 70 °C for 1 h, and the contents were neutralised with 0.2 N HCl (50 μL) after cooling to room temperature. An aliquot of the L-FDAA derivative was dried under vacuum, diluted with MeCN-5% HCOOH in H_2O (1:1) and separated on a Proteo C18 column (25×1.8 mm i.d.) by means of a linear gradient from 10% to 50% aqueous MeCN containing 5% formic acid and 0.05% trifluoroacetic acid, over 45 min at 0.15 mL/min. The RP-HPLC system was connected to the electrospray ion source by inserting a splitter valve and the flow going into the mass spectrometer source was set at a value of 100 μL/min. Mass spectra were acquired in positive ion detection mode (*m*/*z* interval of 320–900) and the data were analyzed using the suite of programs Xcalibur; all masses were reported as average values. The capillary temperature was set at 280 °C, capillary voltage at 37 V, tube lens offset at 50 V and ion spray voltage at 5 V.

All FDAA derivatives of amino acid residues in perthamide E were co-eluted with the corresponding ones in perthamide C.[6]

To determine the absolute configuration of Phe residue in perthamide F, an authentic sample of perthamide D was used as a standard. The hydrolysate of perthamide F contained L-Phe (45.0 min).

3.5. Computational details

Molecular mechanics (MM) calculations were performed using MacroModel 8.5[14] and the MMFFs force field OPLS. MonteCarlo Multiple Minimum (MCMM) method (10,000 steps) was used in order to extensively explore the conformational space. All the structures, so obtained, were optimised using the Polak-Ribiere Conjugated Gradient algorithm (PRCG, 1000 steps, maximum derivative less than 0.05 kcal/mol). The initial geometries of the minimum energy conformers were optimised at the hybrid DFT MPW1PW91 level using the 6-31G(d) basis set (Gaussian 09 software).[16] GIAO ^{13}C and ^{1}H NMR chemical shifts were performed using the MPW1PW91 functional, the 6-31G(d, p) basis set and DMSO as solvent, using as input the geometry previously optimised at MPW1PW91/6-31G(d) level.

3.6. Anti-inflammatory assays

3.6.1. Isolation, culture and stimulation of primary human keratinocytes (PHK). Foreskins from healthy young males were the source of primary human keratinocytes. All protocols and procedures were approved by the Institutional Ethical Committee. Isolation of keratinocytes was performed essentially as described by Boyce and Ham;[18] briefly, skin samples were washed, cut, scraped, and treated with a disperse solution to prepare a largely epidermal layer. The tissue was then trypsinized, filtered, centrifuged, and the resultant keratinocytes cultured and seeded as required. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in a serum-free low-Ca^{2+} (<0.1 mM) defined Keratinocyte-SFM (Invitrogen). The medium was changed every other day. For all experiments, cells were seeded at passage numbers 1–3 into 24-well plates (25×10^5 cells per well) and were treated upon reaching 70–90% confluence. Before the experiments, cells were grown in basal medium without growth factors for 24 h to avoid the effects of supplements in the growth medium and to obtain quiescent cells with low levels of activated NF-κB. Prior to the addition of the stimulus, the basal medium was renewed (300 μL) and the cells were subjected to 30 min pretreatment with perthamide C or perthamide E. 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) (Sigma–Aldrich) 1 μg/mL was then added to the culture and the cells were incubated for 7 h after which the supernatant was removed and stored at 20 °C until further use. The MTT assay was performed to the remaining cells to test the effect of the compounds on cell viability.

3.7. Cytotoxicity evaluation (MTT assay)

Cytotoxicity of the tested compounds was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) uptake method as described.[19] Briefly, at the end of the incubation period (7 h), culture medium was withdrawn. The cell culture was incubated for 1 h at 37 °C and in the dark with 200 μL of MTT at 0.5 mg/mL in basal medium. Formazan violet crystals, induced by MTT cleavage by mitochondrial enzymes, were dissolved in DMSO and analyzed by spectrophotometry at λ=490 nm.

3.8. ELISA assays

Human TNF-α levels in cell supernatants were measured using an ELISA kit from R&D Systems. IL-8 ELISA kit was from Bender MedSystems. Both ELISA analyses were performed in accordance with the manufacturer's instructions.

3.9. Statistical analysis

The results are presented as meanSEM and n represents the number of experiments. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons.

Acknowledgements

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Artículo 7

Potential antipsoriatic effect of chondroitin sulfate through inhibition of NF-kappaB and STAT3 in human keratinocytes

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Potential antipsoriatic effect of chondroitin sulfate through inhibition of NF-κB and STAT3 in human keratinocytes

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ABSTRACT

Chondroitin sulfate (CS) is a natural glycosaminoglycan, formed by the 1–3 linkage of D-glucuronic acid to N-acetylgalactosamine, present in the extracellular matrix. It is used as a slow acting disease modifying agent in the treatment of osteoarthritis, and part of its beneficial effects are due to its antiinflammatory properties that result from an inhibitory effect on NF-κB signaling pathway. This ability raises the hypothesis that CS might be effective in other chronic inflammatory processes such as psoriasis, in which a deregulation of NF-κB is a key feature. In addition, psoriasis is characterized by an upregulation of STAT3 signaling pathway that is related to the epidermal hyperplasia. In the present study we report the pharmacological modulation of the NF-κB and STAT3 signaling pathways by CS in normal human keratinocytes. CS inhibited NF-κB activation and the release of some of the key psoriatic cytokines such as TNFα, IL-8, IL-6 and CCL27. Moreover, it impaired STAT3 translocation to the nucleus and significantly reduced STAT3 transcriptional activity by a mechanism that was independent from STAT3 phosphorylation. Our results confirm the interest of CS as a candidate for future drug research in the therapeutics of psoriasis given the need of more effective and safer oral medications for these patients.

1. Introduction

Psoriasis is a chronic inflammatory skin disorder characterized by inflammation in dermis and epidermis, keratinocyte hyperproliferation, leukocyte infiltration and dilatation and growth of blood vessels. Although the etiology of psoriasis supports a role for genetic and environmental factors, immune-mediated inflammatory processes, involving both innate and adaptive immunity effector mechanisms drive this pathology [1]. Among all the signaling pathways involved in skin inflammation, the transcription factor NF-κB has been described as a crucial player in the pathogenesis of psoriasis. Thus, activation of the NF-κB pathway in psoriatic skin leads to the transcription of numerous genes including cytokines, such as TNFα, chemokines and growth factors, which are involved in the characteristic cutaneous symptomatology of this disease [2]. Activation of NF-κB induces the production of proteins, such as TNFα, which are, in turn, able to stimulate the signal transduction pathway to activate NF-κB, thus conforming a vicious cycle [2]. In fact, TNFα is one of the most important cytokines associated with innate immunity. It controls and regulates the expression of numerous genes, and in doing so leads to cutaneous responses in psoriasis. The intracellular events from the TNFα receptor to NF-κB activation in epidermal keratinocytes have been extensively studied [3,4] and recently developed anti-inflammatory therapies based on blocking TNFα signaling have been shown to be effective in the treatment of psoriasis and could become a highly promising option for this pathology [1,5].

Together with the infiltrated cells, keratinocytes contribute to the chronification of the inflammatory state by producing chemokines, such as IL-8 and CCL27/CTACK (cutaneous T cell-attracting chemokine) in psoriatic plaques, setting up a positive feedback loop which enhances cell recruitment [6,7]. IL-8 stimulates chemotaxis and degranulation of neutrophils, induces angiogenesis and modulates several functions of keratinocytes, including HLA-DR expression and proliferation [6]. CCL27/CTACK is a chemokine constitutively expressed by keratinocyte and highly upregulated in inflammatory skin diseases. This chemokine induces

Abbreviations: CS, chondroitin sulfate; EMSA, electrophoretic mobility shift assay; NHK, normal human keratinocyte; OA, osteoarthritis; STAT, signal transducers and activators of transcription; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; CCL27/CTACK, cutaneous T cell-attracting chemokine.

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inflammation by promoting Th1 and Th2 lymphocyte migration into the skin [7–9] and can be induced in cultured keratinocytes by TNFα, which is also known to induce activity of the transcription factor NF-κB [9,10].

Other cytokines and growth factors upregulated in psoriasis, such as IL-6 and the IL-20 family cytokines, signal through activation of the signal transducer and activator of transcription 3 (STAT3) [11–13]. In fact, increased phosphorylation of STAT3 (pSTAT3) has been observed in lesional skin of psoriatic patients, and transgenic mice with keratinocytes expressing constitutively active STAT3 develop skin lesions strikingly similar to human psoriatic plaques and constitute a mouse model for this pathology [14]. Moreover, STAT3 regulates the expression of genes that mediate survival (survivin, bclxl, mcl-1), proliferation (c-fos, cmyc, cyclinD1), invasion (MMP-2) and angiogenesis (VEGF) through collaboration with other transcription factors, including NF-κB [15].

Chondroitin sulfate (CS) is a natural glycosaminoglycan (GAG) present in the extracellular matrix surrounding cells, especially in the cartilage, skin and blood vessels, where it forms an essential component of proteoglycans. It is formed by the 1–3 linkage of D-glucuronic acid to N-acetylgalactosamine and the disaccharide units are attached by β 1–4 galactosamine links. The galactosamine residues are sulfated either in position 4 (ΔdiA-4S), 6 (ΔdiA-6S) or 4 and 6 (ΔdiA-4,6S). The sulfate groups along with the carboxyl groups of glucuronic acid are ionized, conferring a negative charge that draws water into tissues and hydrates them [16,17].

CS has been shown to be a safe drug with a positive global effect on osteoarthritis (OA) structural changes [18] and improvement of symptoms [19]. The European League Against Rheumatism (EULAR) supports the usefulness of CS in the management of knee osteoarthritis and grants the highest level of evidence to this product coupled with a very low level of toxicity, confirming CS as one of the safest drugs for osteoarthritis treatment [20]. The beneficial effect of CS on patients with OA results from different effects on articular tissues, primarily its immune-modulatory profile, mediated by the inhibition of NF-κB nuclear translocation and the decrease in the production of proinflammatory cytokines such as IL-1β and TNFα [17], among others. These properties raise the hypothesis that CS might be effective in other chronic inflammatory processes in which a deregulation of NF-κB is a key feature such as psoriasis [5].

Preliminary clinical evidence suggesting the possible antipsoriatic effect of CS has been reported. In a series of 11 patients with knee OA and concomitant psoriasis, the use of CS as a symptomatic treatment resulted in a marked clinical and histological improvement of the psoriatic lesions [21]. Furthermore, in a double-blind, placebo-controlled clinical trial, the daily administration of CS for 3 months to patients with knee OA, reduced the incidence of concomitant plantar psoriasis [22]. In light of this evidence, we sought to elucidate the inhibitory effect of CS on several biomarkers of psoriatic skin. In the present study, we show that CS effectively downregulates NF-κB and STAT3 signaling pathways in human primary keratinocytes, limiting, thus, the release of proinflammatory cytokines and chemokines, which have been implicated in the pathogenesis of psoriasis.

2. Materials and methods

2.1. Isolation, culture and stimulation of primary human keratinocytes

All protocols and procedures were approved by the University of Valencia Ethical Committee and were carried out according to the Declaration of Helsinki Principles. Primary human keratinocytes were isolated from foreskins of healthy young donors essentially as described previously [23]. Briefly, skin samples were treated with a dispase solution and trypsinized. The resultant keratinocytes were grown at 37 °C in a humidified atmosphere with 5% CO_2 in a serum-free low-Ca^{2+} (<0.1 mM) defined keratinocyte-SFM (Invitrogen, Carlsbad, CA). The medium was renewed every other day.

For all experiments, cells were seeded at passage numbers 1–3 and treated upon reaching 60–80% confluence. The day before the experiments, medium was replaced to growth factor-free keratinocyte medium (KBM), and the cells were incubated for another 24 h before stimulation. Prior to the addition of the stimulus, KBM was renewed and the cells were subjected to 24 h pretreatment with CS (CS Bio-ActiveTM, Bioibérica S.A., Barcelona, Spain), dissolved in sterile water and added to the culture medium in a ratio of 1% vehicle. Reference molecules such as dexamethasone (1 μM) and the proteasome inhibitor MG-132 (1 μM) (Sigma–Aldrich, St. Louis, MO) or the Jak2 Inhibitor "1,2,3,4,5,6-hexabromocyclohexane" (50 μM) (Calbiochem, San Diego, CA) were preincubated during 30 min in the same conditions. Cells were stimulated with either IL-6 (50 ng/ml) or TNFα (10 ng/ml) from R&D Systems (Abingdon, UK), or 12-*O*-tetradecanoylphorbol-13-acetate (TPA, 1 μg/ml) from Sigma–Aldrich (St. Louis, MO). After the desired stimulation time, supernatants were collected to assess cytokine production and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed in the remaining keratinocytes to assess the possible effects of CS on cell viability [24]. TNFα, CCL27, (R&D Systems, Abingdon, UK) and IL-6, IL-8 (eBioscience, San Diego, CA) levels were assessed using ELISA assays following standard manufacturer protocols. In some experiments, cells were collected in order to obtain the protein extract to assess protein expression.

2.2. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described previously [25]. Cells were washed in ice-cold PBS and then treated with a lysis buffer pH 7.4 (10 mM HEPES, pH 8, 1 mM EDTA, 1 mM EGTA, 10 mM KCl, 1 mM dithiothreitol, 5 mM NaF, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 0.1 μg/ml aprotinine, and 0.5 mM phenylmethyl sulfonyl fluoride) for 15 min, followed by addition of Nonidet P-40 10%. After vigorous vortexing, nuclei were pelleted by centrifugation at 13000 × *g* for 1 min at 4 °C. Nuclear pellets were incubated at 4 °C for 30 min in lysis buffer supplemented with 400 μM NaCl. After centrifugation at 13000 × *g* for 5 min at 4 °C, supernatants were collected. Protein concentration of the nuclear extracts was determined by Bio-Rad DC Protein Assay (Richmond, CA). Double-stranded oligonucleotides containing the consensus NF-κB sequence (Promega Corp., Madison, WI) were end-labeled using T4 polynucleotide kinase (GE Healthcare, Wessling, Germany) and [γ-^{32}P] ATP, followed by purification on G-25 microcolumns (GE Healthcare, Wessling, Germany).

Binding to the consensus sequence was achieved by incubating 10 μg of nuclear extract, 100000 cpm of the labeled probe and 2 μg of poly(dI-dC) in binding buffer (40 mM HEPES pH 8.1, 0.2 mM EDTA, 100 mM NaCl, 2 mM dithiothreitol, 10% (v/v) glycerol) for 20 min at room temperature. Complexes were then analyzed by nondenaturing 6% polyacrylamide gel electrophoresis in 45 mM Tris–borate buffer (pH 8.3) containing 1 mM EDTA, followed by autoradiography of the dried gel using a Typhoon Imager-9400 (GE Healthcare, Wessling, Germany).

2.3. Western blotting

Cell cultures were washed in ice-cold PBS and lysed [25]. The lysates were then sonicated and centrifuged. Supernatants were collected and protein concentrations were determined by

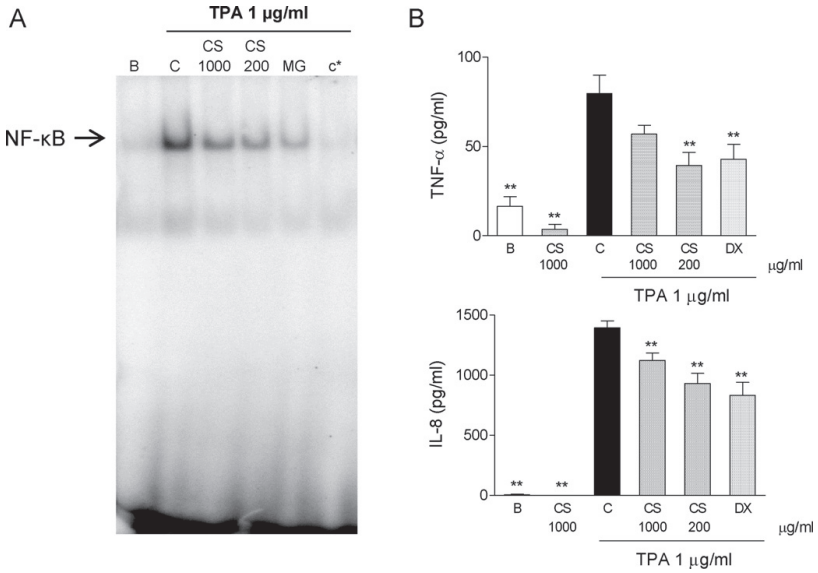

Fig. 1. Inhibitory effect of CS on NF-κB signaling pathway. (A) NF-κB EMSA of nuclear extracts of cultured NHK. Cells were preincubated with CS (1000 or 200 µg/ml, 24 h) or the proteasome inhibitor MG-132 (MG) (1 µM, 30 min) before 1 h TPA stimulation. One out of five independent experiments is shown. (B) Non stimulated cells. (C) Vehicle-treated stimulated cells. (c*) 50-fold excess of unlabeled oligonucleotide. (B) Effect of CS on TNFα and IL-8 release in NHK after 7 h stimulation with TPA. CS was added 24 h prior to the experiments. Dexamethasone (DX, 1 µM) was used as reference compound. Values are expressed as mean ± SEM ($n = 6$) $^{*}p < 0.05$; $^{**}p < 0.01$ *vs* C. (B) Non stimulated cells. (C) Vehicle-treated stimulated cells.

Bio-Rad DC Protein Assay (Bio-Rad, Richmond, CA). The samples were stored at −80 °C until further use. Equal amounts of protein were separated on a 12% SDS-PAGE gel and blotted onto polyvinylidene difluoride Hybond-P membranes (GE Healthcare, Wessling, Germany). Membranes were then blocked in PBS-Tween 20 containing 3% w/v skimmed milk, and incubated with the specific polyclonal antibody against STAT3 or pSTAT3 (Tyr705) (Cell Signaling Technology, Beverly, MA). Primary antibody was detected by enhanced chemioluminescence using horseradish peroxidase conjugated secondary antibodies (Dako, Glostrup Denmark) and a standard ECL substrate (GE Healthcare, Wessling, Germany). Images were captured with the AutoChemi image analyzer (UVP Inc., Upland, CA). β-Actin was used as a protein loading control.

2.4. Immunofluorescence analysis

Cultured primary human keratinocytes were fixed and permeabilized in prechilled absolute methanol for 15 min and rinsed with PBS. Cells were then incubated overnight at 4 °C with polyclonal rabbit anti-STAT3 (Cell Signaling Technology, Beverly, MA). Secondary staining was obtained with Alexa Fluor® 488 secondary antibody (Invitrogen, Carlsbad, CA) for 1 h at room temperature. Finally, samples were washed and nuclear staining was performed by embedding samples in Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). Samples were evaluated by epifluorescence microscopy with a Nikon Eclipse E600FN microscope (Nikon Corp., Tokyo, Japan). As a negative control, sections were incubated with PBS instead of primary antibody.

2.5. Transfection and determination of promoter activity

STAT3 promoter studies were carried out according to manufacturer's instructions. Briefly, human keratinocyte HaCaT cells were cultured in 24-well plates in Dulbecco's modified Eagle's medium supplemented with 10% FBS (Sigma–Aldrich, St. Louis, MO) and transfected at 60–70% confluence. Cells were transfected with 0.5 µg of the STAT3-promoter-luciferase-Renilla reporter plasmids (SABiosciences, QIAGEN, Frederick, MD, USA) using Fugene 6 transfection reagent (Roche Diagnostics, Mannheim, Germany). 5 h after transfection, CS was added for 24 h. Finally, cells were stimulated with IL-6 (50 ng/ml) for 3 h. After stimulation, cells were harvested with passive lysis buffer from Promega, Madison, WI, USA, and Firefly and Renilla luciferase activity were measured by the Dual Luciferase assay system (Promega, Madison, WI, USA) on a Wallac 1420 VICTOR^{2TM} (PerkinElmer, Finland). A positive control expressing both Firefly and Renilla luciferase and two negative controls, each one of them expressing only one of the luciferase activities, were added to all the experiments. Promoter activity was reported as the ratio between Firefly and Renilla luciferase activities in each sample.

2.6. Statistical analysis

Results are presented as mean ± SEM; n represents the number of experiments. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's t-test for multiple comparisons performed by GraphPad Prism 4 software (GraphPad Software, Inc., San Diego, CA). Significance was assumed at a p value of 0.05 or less.

3. Results

3.1. CS inhibits NF-κB activation and reduces TNFα and IL-8 release in TPA-stimulated normal human keratinocytes

The stimulation of NHK with the protein kinase C activator TPA, promotes NF-κB transcriptional activity in keratinocytes,

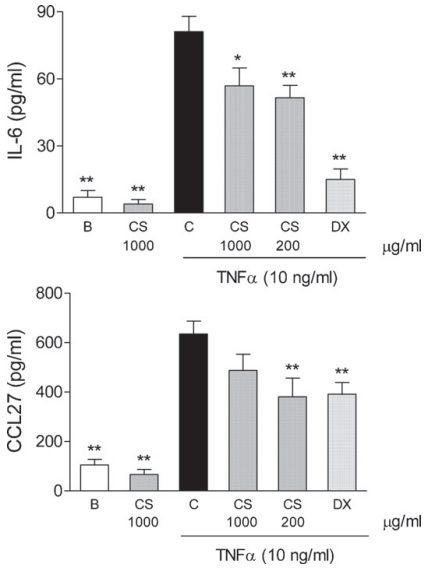

Fig. 2. CS inhibits IL-6 and CCL27 release in TNFα stimulated NHK. CS was added 24 h prior to the experiments. NHK were stimulated during 48 h with TNFα. Dexamethasone (DX, 1 μM) was used as reference compound. Values are expressed as mean ± SEM ($n = 6$) $^{*}p < 0.05$; $^{**}p < 0.01$ *vs* C. (B) Non stimulated cells. (C) Vehicle-treated stimulated cells.

upregulating the transcription of TNFα and other proinflammatory cytokines [26]. In accordance with its demonstrated mechanism of action in other cellular types [17], preincubation with CS impaired NF-κB-DNA binding in the nuclear extracts of 1 h TPA-stimulated NHK (Fig. 1A). This effect was observed at both tested concentrations, 200 and 1000 μg/ml, which are habitually used by other laboratories because provide the physiological range of sulfated glycosaminoglycans [27]. CS pretreatment also reduced TNFα and IL-8 release in 7 h TPA-stimulated NHK (Fig. 1B), demonstrating the ability of CS to decrease the production of cytokines that play a pivotal role in psoriasis pathogenesis [6].

3.2. CS inhibits IL-6 and CCL27/CTACK release in TNFα stimulated human keratinocytes

Once we had established the inhibitory effect of CS on TNFα release by NHK, and given the eminent role played by this cytokine in the pathogenesis of psoriasis, further demonstrated by the effectiveness of biological therapies against this cytokine [6], we next characterized the effect of CS on the release of several cytokines induced by TNFα signaling pathway in NHK. After 48 h of TNFα stimulation, CS inhibited the production of IL-6, cytokine involved in the psoriatic epidermal hyperproliferation [6], and CCL27/CTACK, a skin-specific chemokine expressed by keratinocytes and highly upregulated in inflammatory skin diseases [7] (Fig. 2). CS did not exert cytotoxic effects at the tested concentrations during the 48 h incubation period with NHK, as determined by the MTT reduction assay (data not shown).

3.3. CS inhibits STAT3 nuclear translocation in normal human keratinocytes

It has been postulated that cytokine/growth factor profile associated with psoriasis converges, in part, on Jak/STAT3 signaling in epidermal keratinocytes, being IL-6 one of principal cytokines

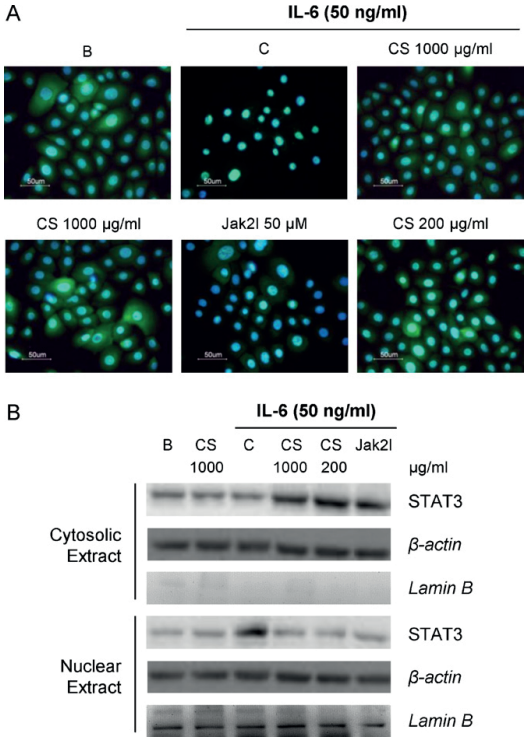

Fig. 3. Inhibitory effect of CS on STAT3 nuclear translocation. (A) Immunofluorescence staining of total STAT3 (Green) in 1 h IL-6-stimulated NHK. Blue color (40,6-diamidine-20-phenylindole dihydrochloride, DAPI) stains cell nuclei. Representative images from three independent experiments. Scale bar = 50 μm. (B) Western blotting of nuclear and cytosolic protein extracts of 1 h IL-6-stimulated NHK using anti-total STAT3 and anti-lamin B, a marker for nuclear protein. Representative images from one out of three experiments. (B) Non stimulated cells. (C) Vehicle-treated stimulated cells. CS was added 24 h prior to the experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

which signal through STAT3 activation [11–13,28]. NHK were stimulated with IL-6 for 1 h after CS pretreatment and STAT3 nuclear translocation was observed by immunofluorescence staining. As shown in Fig. 3, STAT3 was retained in the cytoplasm of CS-treated cells, as well as in the cytoplasm of cells treated with 1,2,3,4,5,6-hexabromocyclohexane, a Jak 2 inhibitor used as reference. This effect was further confirmed by Western blotting analysis of the nuclear and cytosolic protein extracts. STAT3 translocated to the nucleus and thereby was present mainly in the nuclear extracts of the IL-6-stimulated cells, whereas it stayed in the cytoplasm of cells that had been pretreated with either CS or the Jak 2 inhibitor, demonstrating a decrease of STAT3 activation in CS-pretreated cells (Fig. 3B). The nuclear protein lamin B was analyzed in order to assess the purity of the cytosolic and nuclear extracts.

3.4. CS inhibits STAT3 transcriptional activity independently of STAT3 phosphorylation

In order to further assess the effect of CS on STAT3 transcriptional activity, a luciferase assay was performed in HaCaT cells, an immortalized keratinocyte cell line, due to the low transfection efficiencies obtained with NHK. In this assay, CS significantly inhibited STAT3 promoter activity in IL-6-stimulated HaCaT cells,

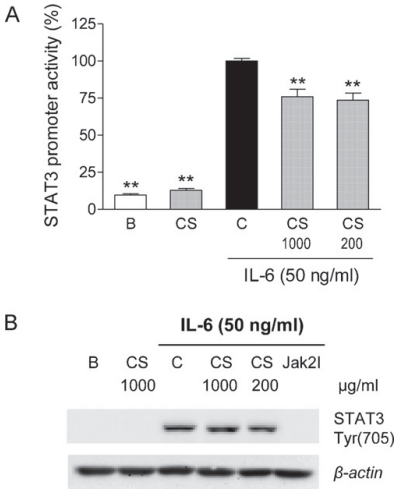

Fig. 4. Inhibitory effect of CS on STAT3 transcriptional activity is independent from STAT3 phosphorylation. (A) HaCaT cells were transfected with STAT3-promoter-luciferase-Renilla plasmids and stimulated with IL-6 for 3 h. Promoter activity was determined as a ratio between Firefly and Renilla luciferase activity. Results are shown as mean ± SEM ($n = 6$). $^{**}p < 0.01$ *vs* C. (B) Western blotting of phospho-STAT3 (Tyr705) in NHK stimulated with IL-6 for 30 min. (B) Non stimulated cells. (C) Vehicle-treated stimulated cells. CS was added 24 h prior to NHK stimulation.

demonstrating the ability of CS to impair STAT3 transcriptional activity (Fig. 4A).

Several studies have identified the phosphorylation of STAT3 in the Tyr705 residue as a critical event for STAT3 nuclear translocation and transcriptional activity in the classical STAT3 activation pathway [15]. In order to deepen our understanding of CS mechanism of action, NHK were incubated with IL-6 for 30 min. However, in contrast to the Jak 2 inhibitor, no effect on STAT3 phosphorylation was observed after CS treatment in NHK (Fig. 4B). These results demonstrate that CS is capable of inhibiting STAT3 nuclear translocation and transcriptional activity through a mechanism that is independent of STAT3 (Tyr705) phosphorylation.

4. Discussion

In the present study, we have demonstrated the ability of CS to impair NF-κB and STAT3 signaling in human keratinocytes. Both transcriptional factors are implicated in the mechanisms that trigger various skin diseases including psoriasis, and their abrogation has been postulated as therapeutic strategy for these pathologies [1,28].

The inhibition of NF-κB nuclear translocation and the consequent reduction of proinflammatory cytokines exerted by CS have been demonstrated in many cellular types such as chondrocytes, synoviocytes, fibroblasts or astrocytes, suggesting that systemic CS may elicit anti-inflammatory effect in many tissues besides the articulation [17,24,29,30]. Our results provide proof of the modulatory effect of CS on human keratinocyte inflammatory response through the inhibition of NF-κB activation as well as the reduction of chemokines, such as IL-8 and CCL27, and cytokines, such as TNFα and IL-6, which play a pivotal role in both the initial stages of lesion formation and the maintenance of the chronic inflammatory state [6,7,31].

A crucial link between high levels of TNFα and NF-κB activation has been found in psoriatic patients, and treatment with biological therapies against TNFα results in downregulation of NF-κB transcriptional activity, which correlates with disease resolution and normalization of the epidermis [5,32]. Therefore, the inhibitory effect of CS on the TNFα/NF-κB positive feedback loop shown in keratinocytes, could in part contribute to the beneficial effect of CS in psoriatic skin. Interestingly, our results are in accordance with previous studies demonstrating the inhibition of TPA-induced NF-κB activation in mouse skin after *in vivo* administration of CS [33].

In psoriatic skin, keratinocytes, together with infiltrating cells, produce chemokines, such as CCL27 and IL-8, which may sustain a cycle of recruitment and inflammation that finally leads to the psoriatic phenotype [1]. Recent studies have shown that p65 binding to the IL-8 promoter is crucial for the transcriptional activation of IL-8 gene [34], and that NF-κB DNA binding to the IL-8 κB site is increased in lesional psoriatic skin compared with non-lesional psoriatic skin [35]. In a similar manner, CCL27 gene expression in NHK is principally regulated through IKKβ/NF-κB signaling pathway [7]. In our study, CS prevents the release of both chemokines, most likely through the inhibition of NF-κB transcriptional activity in stimulated keratinocytes, suggesting that this molecule could exert an inhibitory effect on skin infiltration.

STAT3 is a latent cytoplasmic protein that conveys signal to the nucleus upon stimulation with IL-6 and many other cytokines/growth factors. Classical activation of STAT3 is regulated by phosphorylation of tyrosine 705, leading to its dimerization, translocation into the nucleus and activation of genes related to cell proliferation, migration, survival and oncogenesis [15]. It has been demonstrated that STAT3 has a role in cutaneous inflammatory diseases [36]. In fact, epidermal keratinocytes in psoriatic lesions are characterized by increased levels of cytokines which promote STAT3 activation, suggesting that STAT3 inhibition might result in an effective alternative therapy for psoriasis [28].

Our results have demonstrated the ability of CS to impair STAT3 nuclear translocation and transcriptional activity in human keratinocytes through a mechanism that is independent from STAT3 (Tyr705) phosphorylation. In this way, it is interesting to note that other mechanism such as serine kinases phosphorylation can also mediated STAT3 activation [37]. In addition recent reports have identified importins α1, β1 and Ran to be responsible for STAT3 nuclear trafficking independently from its phosphorylation status [38,39]. Thereby, the potential ability of CS to interfere on the non-canonical ways of STAT3 activation or directly prevent STAT3 nuclear import opens a wide field for future research in order to completely characterize CS mechanism of action in skin.

It is worth noting that the CS concentrations tested in the present study did not show a clear dose–response relationship, as both inhibited in a similar manner NF-κB and STAT3 activation. In addition, the lower concentration (200 μg/ml) appears to provide the best results with respect to the inhibition of cytokines release. Other studies have shown that 200 μg/ml of CS can be more effective than 50 or 1000 μg/ml. A possible explanation is that the physiological range of glycosaminoglycans in some tissues, such as synovial fluid ranges from 80 to 330 μg/ml [27,40,41]. In this sense, it must be taken into account that CS is a glycosaminoglycan presenting a large structural variability, with an average length of 50–100 disaccharides. It is, thus, a hypervariable molecule which can selectively bind to proteins depending of the size and the sulfation pattern. The fact that in our study CS presents similar effect in the range of 200–1000 μg/ml suggests that CS could benefit from a wide therapeutic range, providing beneficial effects in inflammatory skin pathologies after oral administration. In this regard, it should be noted that CS plays an important physiological role in skin cells in addition to its anti-inflammatory and immunomodulatory profile. Alterations in the distribution of CS in psoriatic skin have been described, principally in papillary dermis and in basal keratinocytes, suggesting that treatment of psoriatic lesions with CS might also complement for the local loss of certain CS

epitopes or immobilize proteins, normally bound to CS in normal skin [42].

Recently published meta-analysis has questioned the efficacy and usefulness of CS in the clinical management of OA [43]. This conclusion is based on a minimal clinically important difference in pain reduction between active preparations and placebo and has triggered a highly controversial discussion regarding the selection of published clinical trials for conducting a meaningful meta-analysis. However, more recent reports have demonstrated that CS has a slight to moderate efficacy in the symptomatic treatment of OA, with an excellent safety profile [18,19,44,45]. In the wake of this discussion, the origin of the active ingredients in the prescribed preparations was considered to be the most important factor ensuring quality, and thus safety and efficacy, in particular for CS, due to its extraction from different sources. The CS used in the present study, provided by Bioiberica (Barcelona, Spain), is a highly purified mixture of chondroitin 4 and 6 sulfate of bovine origin in a concentration not less that 98%, with an average molecular weight of 15–16 kDa and a 4 sulfated/6 sulfated ratio of 2, whose anti-inflammatory capabilities have been demonstrated in several *in vitro* studies as well as in clinical trials [22,27,29,46,47].

Although the introduction of new biological therapies has revolutionized the treatment of psoriasis over the past few years, there are still needs for those patients whose psoriasis is not severe enough to warrant their use [48]. Over the years, clinical observation has been a great source of new treatments for psoriasis. The good tolerability and safety aspects of oral CS have been largely documented in OA therapeutics. Furthermore, our results demonstrate that CS is able to impair skin inflammation by inhibiting the activation of NF-κB and STAT3, and subsequently, the release of some of the pivotal components of the psoriatic cytokine network. Therefore, the results obtained in the present study suggest the potential benefit of CS in the therapeutics of psoriasis and support the need of further clinical research on its possible antipsoriatic effect.

Conflict of interest

This work has been supported by Bioibérica, S.A., Barcelona, Spain. No funds were provided for writing this manuscript. Please, note that two of the authors are full employers as Medical Director (J. Vergés) and staff member (M. Herrero) of the Unit of Clinical Research of the Scientific and Medical Department of Bioibérica S.A.

Acknowledgements

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Artículo 8

NF-κB and STAT3 inhibition as a therapeutic strategy in psoriasis: in vitro and in vivo effects of BTH

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NF-κB and STAT3 Inhibition as a Therapeutic Strategy in Psoriasis: *In Vitro* and *In Vivo* Effects of BTH

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Benzo[*b*]thiophen-2-yl-3-bromo-5-hydroxy-5*H*-furan-2-one (BTH) is a simple and interesting synthetic derivative of petrosaspongiolide M, a natural compound isolated from a sea sponge with demonstrated potent anti-inflammatory activity through inhibition of the NF-κB signaling pathway. In the present study, we reported the *in vitro* and *in vivo* pharmacological effect of BTH on some parameters related to the innate and adaptive response in the pathogenesis of psoriasis. BTH inhibited the release of some of the key psoriatic cytokines such as tumor necrosis factorα, IL-8, IL-6, and CCL27 through the downregulation of NF-κB in normal human keratinocytes. Moreover, it impaired signal transducers and activators of transcription3 (STAT3) phosphorylation and translocation to the nucleus, which resulted in decreased keratinocyte proliferation. These results were confirmed *in vivo* in two murine models of psoriasis: the epidermal hyperplasia induced by 12-*O*-tetradecanoylphorbol-13-acetate and the imiquimod-induced skin inflammation model. In both cases, topical administration of BTH prevented skin infiltration and hyperplasia through suppression of NF-κB and STAT3 phosphorylation. Our results confirm the pivotal role of both transcriptional factors in skin inflammation, as occurs in psoriasis, and highlight the potential of small molecules as previously unreported therapeutic agents for the treatment of this skin disease, with BTH being a potential candidate for future drug research.

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INTRODUCTION

Psoriasis is a common immune-mediated inflammatory skin disorder affecting 2–3% of the population. It is characterized by infiltrating leukocytes that release growth factors, cytokines, and chemokines affecting epidermal keratinocyte proliferation and differentiation. Although genetic and environmental factors have a role in the etiology of psoriasis, a common set of effectors are involved in the characteristic manifestations of this skin pathology. Therefore, the onset of psoriatic lesions could be inhibited by abrogation of the transcriptional factors that trigger these mechanisms. This therapeutic strategy has been postulated for signal transducers and activators of transcription3 (STAT3) and NF-κB (Miyoshi *et al.*, 2011; Perera *et al.*, 2012).

Increased phosphorylation of STAT3 (pSTAT3) has been demonstrated in lesional skin of psoriatic patients. Transgenic mice expressing constitutively active STAT3 in their keratinocytes develop skin lesions strikingly similar to human psoriatic plaques (Sano *et al.*, 2005). Moreover, several cytokines and growth factors upregulated in psoriasis, such as IL-6 and the IL-20 family cytokines, signal through STAT3 activation (Grossman *et al.*, 1989; Sa *et al.*, 2007; Wolk *et al.*, 2009). STAT3 regulates the expression of genes controlling survival, proliferation, and angiogenesis through collaboration with other transcription factors, including NF-κB (Aggarwal *et al.*, 2009). In addition, STAT3 has been shown to have a role in the psoriasis-associated IL-23 signaling pathway (Di Cesare *et al.*, 2009).

NF-κB is a crucial factor in the immune-inflammatory responses implicated in various skin diseases, including psoriasis. In fact, several antipsoriatic drugs act in part by inhibition of this nuclear factor (Perera *et al.*, 2012). Furthermore, upregulation of NF-κB activation in lesional psoriatic skin compared with nonlesional psoriatic skin has been demonstrated (Lizzul *et al.*, 2005). Activation of the NF-κB pathway leads to the transcription of numerous genes including cytokines, chemokines, and growth factors that are involved in the initiation of the inflammatory response (Perera *et al.*, 2012). Remarkably, NF-κB induces the production of cytokines, such as tumor necrosis factorα (TNFα), which further activate NF-κB, thus conforming a vicious cycle (Quivy and Van Lint, 2004). In this way, an essential link between TNFα levels and NF-κB activation has been found in

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This work was conducted in Valencia, Spain.

Abbreviations: BTH, 4-benzo[b]thiophen-2-yl-3-bromo-5-hydroxy-5H-furan-2-one; CCL27/CTACK, cutaneous T cell–attracting chemokine; IMQ, imiquimod; MPO, myeloperoxidase; NHK, normal human keratinocyte; STAT, signal transducers and activators of transcription; TNFα, tumor necrosis factorα; TPA, 12-O-tetradecanoylphorbol 13-acetate

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psoriatic patients, and disease resolution after treatment with TNFα-blocking agents correlated with downregulation of NF-κB transcriptional activity (Gottlieb *et al.*, 2005; Lizzul *et al.*, 2005).

Bioactive natural products are considered to be promising prototypes for the development of new therapeutic agents, as they are evolutionary selected and basically validated for interfering with biological targets. Hence, libraries designed and synthesized around the basic structure of such compounds have a good chance of displaying biological and pharmacological properties. On the basis of these remarks, a series of analogs containing the γ-hydroxybutenolide moiety of the marine natural compound petrosaspongiolide M, a potent anti-inflammatory agent that inhibits NF-κB activation (Posadas *et al.*, 2003), were synthesized (Guerrero *et al.*, 2007; De Simone *et al.*, 2010). Among them, the synthetic derivative 4-benzo[*b*]thiophen-2-yl-3-bromo-5-hydroxy-5*H*-furan-2-one (BTH) (Figure 1a) was characterized as a potent NF-κB inhibitor *in vitro* (Guerrero *et al.*, 2007), which exerted an anti-inflammatory effect in both the acute murine air pouch model and the chronic collagen-induced arthritis (Guerrero *et al.*, 2009).

Given the interesting anti-inflammatory profile and the strong inhibition of NF-κB activation elicited by BTH, we hypothesized that it might be effective in other chronic inflammatory diseases such as psoriasis. In the present study, we describe the pharmacological effect of BTH on the activation and proliferation of human keratinocytes and its efficacy after topical application in the murine models of 12-*O*-tetradecanoylphorbol 13-acetate (TPA)–induced hyperplasia and imiquimod (IMQ)-induced skin inflammation. The potential antipsoriatic profile of BTH can be a consequence of its inhibitory effect on the NF-κB and STAT3 pathways.

RESULTS

BTH inhibits NF-κB activation and proinflammatory cytokine release in normal human keratinocytes

Our first goal was to assess whether BTH maintained its mechanism of action in normal human keratinocytes (NHKs) stimulated with the protein kinase C activator TPA, which promotes NF-κB transcriptional activity, upregulating the transcription of TNFα and other proinflammatory cytokines (Cataisson *et al.*, 2005). Preincubation for 30 minutes with either BTH (1 and 10 μM) or the proteasome inhibitor MG-132 (1 μM) inhibited NF-κB-DNA binding in NHKs stimulated with TPA (1 μg ml^{-1}) for 1 hour (Figure 1b). Consequently, BTH also reduced the release of TNFα and IL-8, cytokines that have a pivotal role in psoriasis pathogenesis (Pietrzak *et al.*, 2008), after 7 hours of stimulation with TPA (Figure 1c).

We next characterized the effect of BTH on the release of several cytokines induced by TNFα (10 ng ml^{-1}). In 48 hours–stimulated NHKs, BTH inhibited the production of IL-6, a cytokine involved in the psoriatic epidermal hyperproliferation, and CCL27/CTACK (cutaneous T cell–attracting chemokine), a keratinocyte-specific chemokine highly upregulated in inflammatory skin diseases (Riis *et al.*, 2011) (Figure 1d).

BTH impairs STAT3 translocation to the nucleus and decreases keratinocyte proliferation *in vitro*

As shown in Figure 2a and b, an inhibition of IL-6-induced STAT3 nuclear translocation was observed by immunofluorescence staining after a 30-minute pretreatment with BTH.

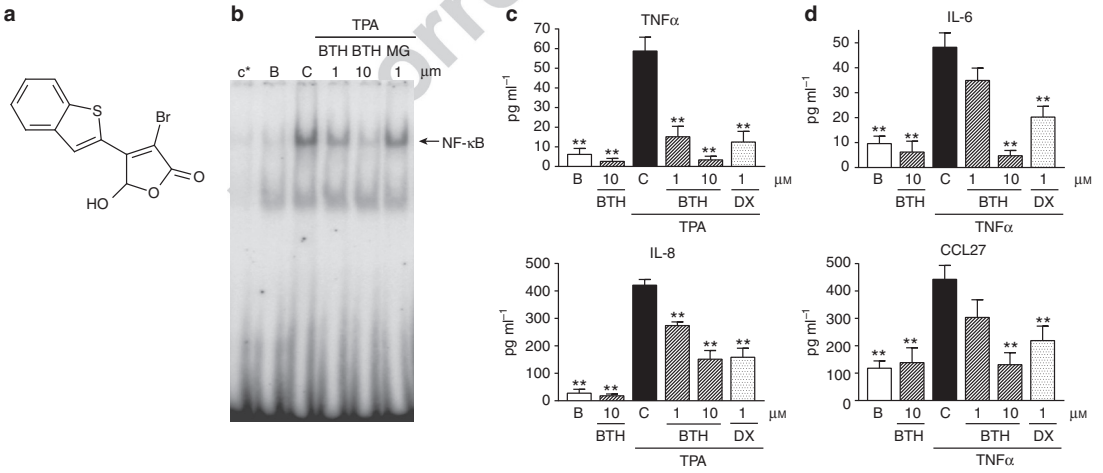

Figure 1. Inhibitory effect of 4-benzo[*b*]thiophen-2-yl-3-bromo-5-hydroxy-5*H*-furan-2-one (BTH) on NF-κB activation and cytokine release in normal human keratinocytes (NHKs). (**a**) Chemical structure of BTH. (**b**) NF-κB electrophoretic mobility shift assay of the nuclear extracts of cultured NHKs. Cells were preincubated with BTH or the reference compound MG-132 (MG) for 30 minutes before 1 hour of 12-*O*-tetradecanoylphorbol-13-acetate7 (TPA) stimulation (1 μg ml^{-1}). One out of five independent experiments is shown. (**c**) Effect of BTH on tumor necrosis factorα (TNFα) and IL-8 release in NHKs after 7 hours of stimulation with TPA (1 μg ml^{-1}). (**d**) Effect of BTH on IL-6 and CCL27 release in NHKs after 48 hours of stimulation with TNFα (10 ng ml^{-1}). Dexamethasone (DX, 1 μM) was used as reference compound. Values are expressed as mean ± SEM ($n = 6$) $^{**}P < 0.01$ versus C. B, nonstimulated cells; c*, 50-fold excess of unlabeled oligonucleotide; C, vehicle-treated stimulated cells.

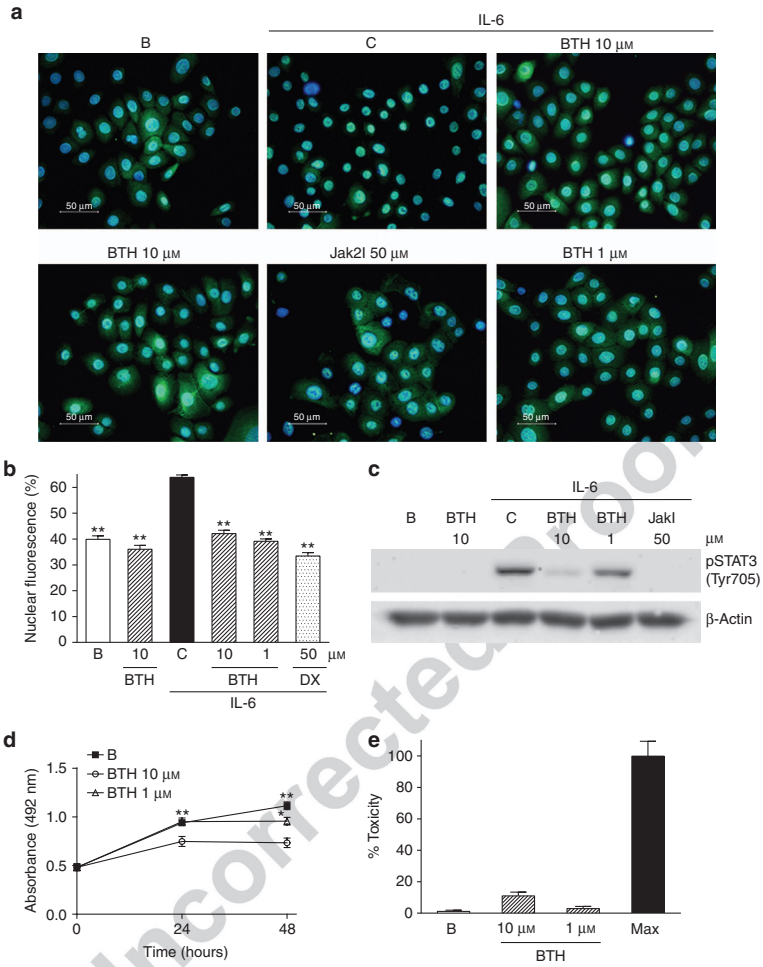

Figure 2. 4-Benzo[*b*]thiophen-2-yl-3-bromo-5-hydroxy-5*H*-furan-2-one (BTH) impairs keratinocyte proliferation through inhibition of STAT3 phosphorylation. (**a**) Immunofluorescence staining of total STAT3 (green) in 1-hour IL-6 (50 ng ml^{-1})-stimulated normal human keratinocytes (NHKs). Blue color (4′6-diamidino-2-phenylindole) stains cell nuclei. Representative images from three independent experiments. (**b**) Quantitative analysis of the STAT3 intracellular localization. Results are expressed as % of total fluorescence per cell ($n=15$) $^{**}P<0.01$, versus C. (**c**) Western blotting of phospho-STAT3 in NHKs stimulated with IL-6 (50 ng ml^{-1}) for 30 minutes. One out of three independent experiments is shown. (**d**) NHK proliferation measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Data are mean absorbance at 490 nm ± SEM ($n=9$) $^{*}P<0.05$, $^{**}P<0.01$, versus B (vehicle treated). (**e**) % toxicity determined as lactate dehydrogenase levels in the supernatants of NHKs cultured for 48 hours with BTH. NHKs in vehicle alone (B) or with Triton X-100 (Max) are set as 0% and 100% of cytotoxicity, respectively. Data are mean ± SEM ($n=9$). B, nonstimulated cells; C, vehicle-treated stimulated cells. Bar = 50 μm.

In contrast to the control stimulated cells, STAT3 was retained in the cytoplasm of BTH-treated cells, as well as in the cytoplasm of cells treated with 1,2,3,4,5,6-hexabromocyclohexane (50 μM), a Jak 2 inhibitor used as reference. In addition, BTH inhibited the phosphorylation of STAT3 tyrosine 705 (Tyr705) residue in IL-6-stimulated NHKs, as shown by western blotting analysis (Figure 2c). This phosphorylation, carried out by the Jak proteins, is essential for STAT3 dimerization and subsequent nuclear translocation and DNA binding (Aggarwal *et al.*, 2009). Therefore, this could be the mechanism by which BTH inhibits STAT3 translocation.

STAT3 is a key transcriptional factor involved in the regulation of cell proliferation (Aggarwal *et al.*, 2009). In accordance with its capability to inhibit STAT3 activation, BTH impaired keratinocyte growth for 48 hours as observed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction method (Figure 2d). In these conditions, BTH did not modify lactate dehydrogenase levels in cells

supernatants in comparison with untreated cells (Figure 2e), thus ruling out cytotoxicity as the cause of the inhibition in cell proliferation.

BTH inhibits skin inflammation and hyperplasia in the murine TPA–induced model

We evaluated the effect of BTH on the murine TPA-induced epidermal hyperplasia model that reproduces certain biochemical and histopathological parameters characteristic of human psoriatic lesions (Sato *et al.*, 2004; Amigo *et al.*, 2007). In this model, TPA induces pronounced skin inflammation characterized by epidermal hyperplasia, edema, cell infiltration, increased angiogenesis, and high production of cytokines such as TNFα, IL-6, IL-1β, and CXCL-1 (the murine functional analog of IL-8) (Hvid *et al.*, 2008). In addition, the eicosanoids prostaglandin E_2 and leucotriene B_4 are reported to have an important role in the TPA-induced skin inflammation (Murakawa *et al.*, 2006).

Topical treatment with BTH (200 and 400 μg per site) 30 minutes before TPA application (2 nmol per site for three consecutive days) almost completely inhibited lesion formation in a dose-dependent manner. In fact, the higher dose of BTH (400 μg per site) was as effective as the reference topical treatment with dexamethasone (200 μg per site) (Figure 3a).

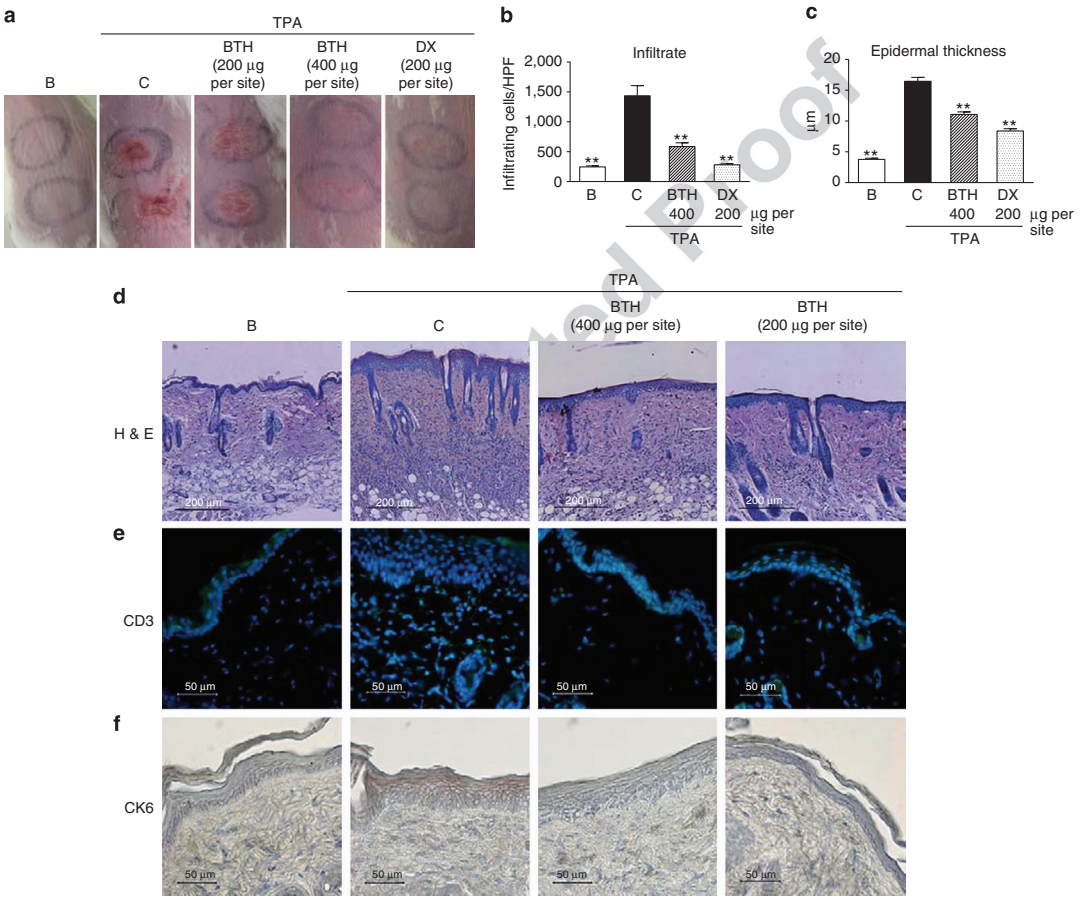

Figure 3. Normalization of epidermal hyperplasia and inflammatory cell infiltration after 4-benzo[*b*]thiophen-2-yl-3-bromo-5-hydroxy-5*H*-furan-2-one (BTH) pretreatment in 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced skin inflammation. BTH, dexamethasone (DX), or vehicle (acetone) were topically applied 1 hour before TPA administration (2 nmol per site) during 3 consecutive days. (**a**) Macroscopic appearance of the skin at the end of the experiment. (**b**) Number of infiltrating cells in representative high-power fields (HPFs) and (**c**) epidermal thickness of hematoxylin and eosin (H&E)–stained tissue sections as mean ± SEM ($n = 6$) $^{**}P < 0.01$ versus C. (**d**) Photomicrographs of H&E staining of skin biopsies. Bar = 200 μm. (**e**) Immunofluorescence staining of CD3-positive cells (green, arrows) in skin biopsies. Blue color (4′6-diamidino-2-phenylindole) stains cell nuclei. Bar = 50 μm. (**f**) Immunohistochemical detection of cytokeratin 6 (CK6, brown) protein in mouse skin. Bar = 50 μm. All images are representative from one out of three mice investigated. B, acetone-treated mice; C, acetone and TPA-treated mice.

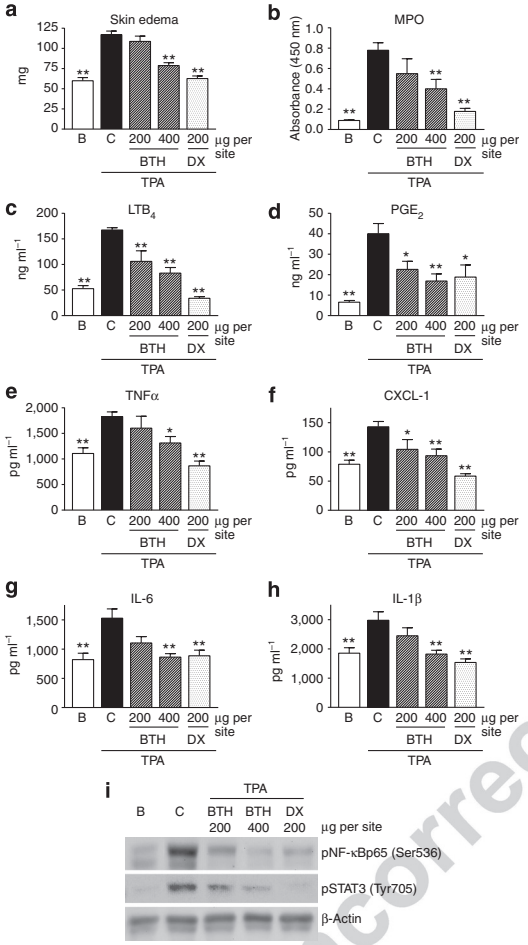

Figure 4. Topical treatment with 4-benzo[*b*]thiophen-2-yl-3-bromo-5-hydroxy-5*H*-furan-2-one (BTH) attenuates 12-*O*-tetradecanoylphorbol-13-acetate (TPA)–induced inflammation and hyperplasia through NF-κB and STAT3 inhibition. BTH, dexamethasone (DX), or vehicle (acetone) were topically applied 1 hour before TPA administration (2 nmol per site) during 3 consecutive days. (**a**) Skin edema, assayed as punch biopsy weight. (**b**) Myeloperoxidase (MPO) activity, (**c**) leucotriene B_4 (LTB_4), (**d**) prostaglandin E_2 (PGE_2), (**e**) tumor necrosis factorα (TNFα), (**f**) CXCL-1, (**g**) IL-6, and (**h**) IL-1β levels determined in skin homogenates. Data represent mean ± SEM ($n = 6$ animals). $^*P < 0.05$, $^{**}P < 0.01$ versus C. (**i**) NF-κB and STAT3 phosphorylation was assessed by western blotting on protein extracts from skin homogenates. One out of three mice investigated are shown. B, acetone-treated mice; C, acetone and TPA-treated mice.

This beneficial effect was further confirmed by the lower weight of the 1-cm^2 punch biopsies taken from BTH-treated mice compared with control TPA-treated mice, suggesting an inhibition of skin edema (Figure 4a).

The histological analysis of lesion skin biopsies by hematoxylin and eosin staining showed a decrease of inflammatory cell infiltrate in BTH-treated mice compared with TPA-treated mice (Figure 3b and d), which correlated with a reduction of myeloperoxidase (MPO) activity and leucotriene B_4 levels in skin homogenates (Figure 4b and c). In addition, BTH was able to diminish not only the nonlymphocytic infiltrate consisting mainly of neutrophils (Sato *et al.*, 2004) but also the $CD3^+$ T-cell infiltrate, as demonstrated by fluorescent immunohistochemistry (Figure 3e, arrows). Furthermore, BTH treatment prevented the characteristic epidermal hyperplasia (Figure 3c and d) and high cytokeratin 6 staining (Figure 3f) induced by TPA, confirming the antiproliferative profile exhibited by BTH *in vitro*.

Topical application of BTH reduced, in a dose-dependent manner, the levels of TNFα, IL-6, and CXCL-1 in skin homogenates, in agreement with the results observed in the *in vitro* study. This effect was accompanied by a clear inhibition of prostaglandin E_2 and IL-1β levels, further suggesting the ability of this compound to reduce inflammatory mediators induced by TPA and regulated by NF-κB activation (Tak and Firestein, 2001; Petersen, 2006) (Figure 4c–h).

Finally, western blotting analysis of skin homogenates showed a TPA-induced STAT3 Tyr705 phosphorylation, which was dose dependently inhibited by topical treatment with BTH. In a similar manner, p65-phosphorylated NF-κB was decreased in skin homogenates of BTH-treated mice compared with control animals (Figure 4i). These results confirm the interesting pharmacological profile of BTH as a potential agent to treat immune disease with an inflammatory component when applied topically to whole skin.

BTH ameliorates IMQ-induced psoriasis-like skin inflammation

We further studied the potential beneficial effect of BTH in the murine IMQ-induced psoriasis-like skin inflammation model in which topical application of IMQ, a Toll-like receptor 7 and 8 agonist, to the whole back of the mice during 6 days induces skin inflammation accompanied by structural features characteristic of psoriasis (van der Fits *et al.*, 2009). In these conditions, BTH pretreatment of a delimited area of 3 cm^2 significantly reduced the development of desquamation and erythema at the site of application, in contrast to the rest of the IMQ-treated skin where desquamation an redness was as marked as in control mice (Figure 5a and b), suggesting that BTH exerts a local topical effect. Histological analysis of punch biopsies showed a reduction of epidermal thickening in BTH-treated skin, as seen with hematoxylin and eosin and BrdU staining (indicative of proliferative keratinocytes) (Figure 5c, d and e). As expected, topical treatment with the reference compound dexamethasone also ameliorated all the above-mentioned parameters. However, in contrast to BTH, the effect of dexamethasone was extensive to the skin beyond the delimited 3-cm^2 application site, and skin atrophy was visible throughout the whole back (Figure 5a).

BTH pretreatment also reduced the dermal infiltrate present in IMQ-treated mice (Figure 5c and f). This effect was further confirmed by the lower MPO activity observed in skin homogenates (Figure 5g). Moreover, BTH significantly decreased IL-23 levels in skin (Figure 5h), a cytokine that has been demonstrated to be essential for the development of

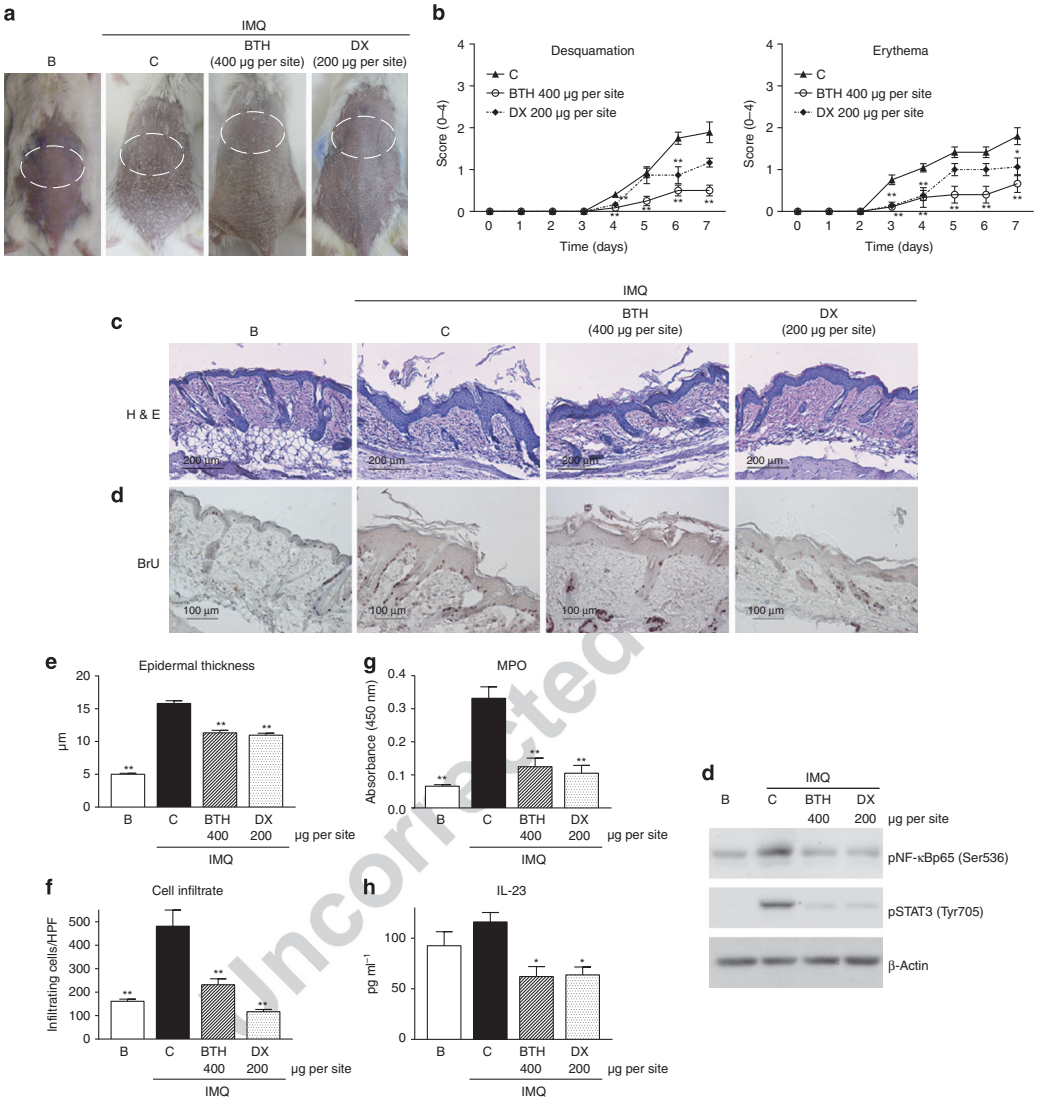

Figure 5. 4-Benzo[*b*]thiophen-2-yl-3-bromo-5-hydroxy-5*H*-furan-2-one (BTH) ameliorates imiquimod (IMQ)-induced skin inflammation. BTH, dexamethasone (DX) or vehicle (acetone) were topically applied in a delimited area of skin (3 cm^2) 1 hour before IMQ administration. (**a**) Phenotypical presentation of mouse back skin after 6 days of treatment. (**b**) Erythema and scaling of the skin was scored daily on a scale from 0 to 4. (**c**) Hematoxylin and eosin (H&E) staining of skin biopsies. Bar = 200 μm. (**d**) BrdU incorporation in keratinocytes was detected by immunohistochemistry. Bar = 100 μm. (**e**) Epidermal thickness and (**f**) number of infiltrating cells in representative high-power fields (HPFs). (**g**) Myeloperoxidase (MPO) activity and (**h**) IL-23 levels determined in skin homogenates. (**i**) NF-κB and STAT3 phosphorylation in skin homogenates. All images are representative from one out of three mice investigated. Data represent mean ± SEM ($n = 6$ animals) $^*P<0.05$, $^{**}P<0.01$ versus C. B, vehicle-treated mice; C, vehicle- and IMQ-treated mice.

this animal model (van der Fits *et al.*, 2009). Finally, western blotting analysis of skin homogenates showed that BTH was able to impair STAT3 (Tyr705) and p65-NF-κB (Ser 536) phosphorylation induced after IMQ treatment (Figure 5i), corroborating the previously described mechanism of BTH.

To conclude, mice spleens were weighed at the end of the experiment to provide a general perception on the immunological status of the animals. As expected, the administration of IMQ produced a marked spleen hypertrophy (van der Fits *et al.*, 2009), which was unaltered after topical BTH treatment

(Supplementary Figure S1 online), further suggesting a local effect of BTH. In contrast, spleen mass from dexamethasone-treated mice was 3-fold lower than that of healthy mice, demonstrating systemic absorption and immunosuppressive effect even after topical application.

DISCUSSION

Psoriasis is a chronic inflammatory skin disease characterized by leukocyte infiltration in the dermis and epidermis, keratinocyte hyperproliferation, and dilatation and growth of blood vessels. Despite the variable course and manifestations of the pathology, the hallmark of the skin lesions is one of inflammation, involving both innate and adaptive immunity effector mechanisms (Sano *et al.*, 2005; Perera *et al.*, 2012). The introduction of new biological therapies has revolutionized the treatment of psoriasis and has helped the understanding of the molecular mechanisms involved in the pathogenesis of this disease. However, biologics do not cover the needs of those patients whose psoriasis is not severe enough to warrant their use. These patients will greatly benefit from better topical treatments or more effective and safer oral medications (Williams, 2012).

In the present study, we sought to determine the antipsoriatic properties of BTH, a synthetic anti-inflammatory agent, bearing the γ-hidroxybutenolide moiety and derived from a natural compound isolated from a sea sponge petrosaspongiolide M. BTH was selected on the basis of its ability to inhibit NF-κB activation and ameliorate collagen-induced arthritis in mice (Guerrero *et al.*, 2007, 2009).

Psoriatic plaques are characterized by a chronic immune response. In this context, keratinocytes, together with the infiltrating cells, produce chemokines such as CCL27 and IL-8, which recruit more leukocytes, setting up a positive feedback loop (Perera *et al.*, 2012). CCL27 binds to CCR10, expressed by more than 90% of skin-infiltrating lymphocytes (Riis *et al.*, 2011), and IL-8 stimulates chemotaxis and degranulation of neutrophils, induces angiogenesis, and modulates HLA-DR expression and proliferation in keratinocytes (Pietrzak *et al.*, 2008). The expression of both chemokines is mediated by NF-κB (Tak and Firestein, 2001; Riis *et al.*, 2011), thereby suggesting a pivotal role of this transcription factor in the inflammatory events driving the conversion of pre-psoriatic skin to psoriatic plaques. Our results demonstrate that BTH strongly prevents the release of both chemokines, most likely through the inhibition of NF-κB transcriptional activity in stimulated keratinocytes, suggesting that this molecule could exert an inhibitory effect on skin infiltration. This hypothesis was confirmed by the *in vivo* studies, in which topical administration of BTH decreased edema and leukocyte recruitment in mouse skin. In this regard, immunofluorescence staining showed a reduction in $CD3^+$ T-cell infiltrate. In addition, the decrease in CXCL-1 and leucotriene B_4 levels, as well as MPO activity, in skin homogenates of BTH-treated mice was consistent with reduced recruitment of neutrophils, which constitute the predominant infiltrating cell type in the TPA-induced hyperplasia model (Ikai, 1999; Sato *et al.*, 2004). One of the primary events in developing psoriatic lesions is the perivascular accumulation of neutrophils and their influx into the epidermis (Bos *et al.*,2005). In these preliminary stages, their phagocytic reactions cause oxidative damage that initiates the expression of redox sensitive transcription factors such as NF-κB (Briganti and Picardo, 2003). Furthermore, neutrophils and mast cells have been recently identified as the major source of IL-17 in human skin (Lin *et al.*, 2011). Therefore, BTH could impair skin inflammation in psoriatic plaques at the early stages by blocking epidermal activation dependent on neutrophils. These results were corroborated by the decrease in cell infiltration and MPO activity observed in the IMQ model.

TNFα is among the best-characterized inducers of NF-κB activity, and a crucial link between high levels of TNFα and NF-κB activation has been found in psoriatic patients (Lizzul *et al.*, 2005). It has been postulated that TNFα produced locally in psoriatic lesions creates a positive feedback loop that amplifies and sustains the inflammatory process within plaques (Banno *et al.*, 2004; Quivy and Van Lint, 2004). BTH inhibited both NF-κB activation and TNFα release *in vitro* and *in vivo*, suggesting an inhibitory effect on this feedback loop. In fact, the downregulation of this transcriptional factor may be the cause of the decrease of all the other cytokines measured in skin samples, as their transcription is dependent on NF-κB activation (Perera *et al.*, 2012). It is interesting to note that NF-κB is a major contributor to the production of chemotactic molecules by keratinocytes even when TNFα is downregulated (Cataisson *et al.*, 2005). Therefore, BTH could provide a therapeutic benefit in skin inflammatory disorders preventing leukocyte accumulation when TNFα antagonists are ineffective.

STAT3 is a main player in cutaneous inflammatory diseases, as well as in normal keratinocyte function (Sano *et al.*, 2005). Increased phosphorylation of STAT3 has been observed in lesional skin and many of the proinflammatory cytokines involved in the pathogenesis of psoriasis, such as IL-6 and the IL-20 subfamily, signal through STAT3. As a consequence, it has been postulated that the cytokine/growth factor profile associated with psoriasis converges, in part, on Jak/STAT3 signaling in epidermal keratinocytes (Miyoshi *et al.*, 2011). Actually, several Jak inhibitors are being tested as new possible therapeutic agents for the treatment of this skin disease with promising results (Boy *et al.*, 2009; Chang *et al.*, 2009; Fridman *et al.*, 2011). Our *in vitro* studies show the ability of BTH to impair STAT3 nuclear translocation by blocking its phosphorylation in the Tyr705 residue. These results suggest that BTH could act through Jak inhibition, as Jak proteins are the main conductors of this modification after a cytokine challenge (Aggarwal *et al.*, 2009). As cytokine receptors can recruit two Jak family members (Jak1, Jak2, Jak3, or Tyk2) into signaling complexes, further studies are needed in order to completely characterize the mechanism and specificity of BTH at this level. In addition, BTH inhibited IL-6 release, one of the main STAT3 activators, after a 48-hour TNFα challenge. In addition, it impaired keratinocyte proliferation *in vitro* and in both murine models tested, as seen by the decreased epidermal thickness and lower expression of cytokeratin 6 and BrdU incorporation in skin sections. It is worth noting that this effect on the epidermal turnover *in vivo*

could be due to the decreased inflammatory milieu apparent from the lower levels of mediators determined on skin biopsies.

Recently, topical administration of IMQ, a ligand for Toll-like receptors 7 and 8, was reported as a novel mouse model of psoriasis (van der Fits *et al.*, 2009). In this model, highly dependent on IL-23, BTH was able to ameliorate the course of the disease, resulting in normalization of skin inflammation and keratinocyte proliferation. More interestingly, in addition to its inhibitory effect on NF-κB and STAT3 activation, BTH was able to reduce IL-23 levels in skin homogenates. The pivotal role of this cytokine, which drives the development of Th17 cells, in the pathogenesis of psoriasis has been robustly confirmed by the success of the highly efficient biological therapies targeted against IL-23 (van der Fits *et al.*, 2009; Yeilding *et al.*, 2012). As STAT3 is the main factor in the IL-23 signaling pathway (Di Cesare *et al.*, 2009), the reduction of this cytokine by BTH could contribute to the inhibitory effect of this compound on STAT3 activation *in vivo*, providing insights to further investigate the effect of this molecule in other cellular types involved in the IL-23/Th17 axis.

Given the acute nature of the animal models used in the present study, we cannot fully predict the effectiveness of BTH on the chronic treatment of established psoriatic plaques. However, the ability of BTH to abolish NF-κB and STAT3 signaling and to decrease IL-23 levels suggests that it could also be beneficial in the treatment of the chronic disease, as these are some of the main orchestrators of the immune response that sustains the chronic psoriatic process. Furthermore, the fact that BTH seems to exert its beneficial effect locally in the area of application could provide a therapeutic advantage with respect to the systematically immunosuppressive effect of topical dexamethasone. However, further studies will be needed to confirm the complete pharmacokinetic profile of this compound after topical application.

In conclusion, the results of our study suggest that BTH would be suitable for epicutaneous application, as topical administration is often more suitable for the treatment of mild-to-moderate psoriasis. We have demonstrated that this synthetic compound diminishes two major signaling cascades involved in the pathogenesis of autoimmune skin diseases. Our data suggest that BTH efficiently penetrates the epidermis, where it blocks NF-κB and STAT3 phosphorylation, leading to normalization of lesional skin inflammation and hyperplasia. The inhibition of both signaling pathways by BTH in the skin could be responsible for the decrease in the production of proinflammatory cytokines, chemokines, and adhesion molecules known to have a role at both the initial stages of lesion formation and the maintenance of the chronic inflammatory state. In addition, the pleiotropic effect of BTH could be of special interest, and thus it affects multiple cell types providing a potential advantage over antibodies targeted against specific cytokines or cell types. In summary, we conclude that BTH has a great potential as an antipsoriatic agent for the treatment of inflammatory skin diseases. Moreover, the interesting pharmacological profile and singular structural features of BTH make this compound a promising candidate for future drug research.

MATERIALS AND METHODS

Isolation, culture, and stimulation of primary human keratinocytes

Foreskins from healthy young donors were the source of primary human keratinocytes. All protocols and procedures were approved by the local ethics committee and carried out according to the Declaration of Helsinki Principles. Patient consent for experiments was not required because Spanish laws consider human tissue left from surgery as discarded material. Isolation of keratinocytes was performed by sequential digestion with dispase and trypsin, as described previously (Andres *et al.*, 2013). Cells were grown in Defined Keratinocyte-SFM (Invitrogen, Carlsbad, CA) and were used between passages 1 and 3. Before the experiments, the medium was replaced with growth factor–free keratinocyte medium, and the cells were incubated for 24 hours before stimulation.

Before the addition of the stimulus, keratinocyte medium was renewed and the cells were subjected to a 30-minute pretreatment with BTH (Cayman Chemical, Ann Arbor, MI) or reference molecules such as dexamethasone (1 μM), MG-132 (1 μM) (both from Sigma-Aldrich, St Louis, MO), and a Jak2 Inhibitor ''1,2,3,4,5,6-hexabromocyclohexane'' (50 μM) (Calbiochem, San Diego, CA). Controls contained an equal volume of vehicle (absolute ethanol, 1% vol/vol). Finally, cells were stimulated with either IL-6 (50 ng ml^{-1}) or TNFα (10 ng ml^{-1}) from R&D Systems (Abingdon, UK) or TPA (1 μg ml^{-1}) from Sigma-Aldrich.

Determination of cytokine release and NF-κB/STAT3 activation *in vitro*

After the specified agent treatment and stimulation time, supernatants were collected and the levels of CCL27, TNFα (R&D Systems), IL-6, and IL-8 (eBioscience, San Diego, CA) were measured using ELISA assays according to the standard manufacturer protocols. In addition, cells were lysed in order to obtain nuclear extracts where NF-κB–DNA binding was assessed by electrophoretic mobility shift assay, as described previously (Andres *et al.*, 2013). Parallely, total cell lysates were obtained and western blotting was performed with a specific antibody against pSTAT3 Tyr705 (Cell Signaling Technology, Beverly, MA), as described elsewhere (Andres *et al.*, 2013). In another set of experiments, cells were fixed, permeabilized, and inmunostained using polyclonal rabbit anti-STAT3 (Cell Signaling Technology) followed by Alexa Fluor 488 secondary antibody (Invitrogen) (Andres *et al.*, 2013). Detailed description of these procedures can be found in Supplementary Materials online.

Cell proliferation and cytotoxicity assay

Keratinocytes were grown in complete keratinocyte growth medium in the presence of BTH for 48 hours. Cell density was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide uptake method as previously described (Borenfreund *et al.*, 1988). Product toxicity was assessed by measuring lactate dehydrogenase release in the culture supernatants (Miyoshi *et al.*, 2011).

Animals

Mice were obtained from Janvier (Le Genest St Isle, France). All studies were conducted in accordance with European Union regulations for the handling and use of laboratory animals. Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Valencia.

TPA-induced epidermal hyperplasia model

A total of 20 µl of 100 µM TPA (2 nmol per site) or vehicle (acetone) was applied to 1 cm^2 of the shaved dorsal area of female Swiss CD-1 mice (25–30 g) using a micropipette. At 1 hour before the inflammatory challenge, BTH (200 or 400 µg per site), dexamethasone (200 µg per site), or vehicle (acetone) was topically administered to the same area. This procedure was repeated during three consecutive days. At the end of the experiment (day 4), mice were killed by cervical dislocation and 1-cm^2 punch biopsies were taken from the treated dorsal skin and weighed in order to measure edema (Amigo *et al.*, 2008). Biopsies were then fixed in formalin for histological study as described previously (Blumberg *et al.*, 2010). Alternatively, biopsies were frozen in liquid N2 and homogenized. Cytokines in skin homogenates were measured by ELISA according to the manufacturer's specifications, and eicosanoids and MPO were determined as described previously (Amigo *et al.*, 2007). Full description of these procedures can be found in Supplementary Materials online.

IMQ-induced psoriasis-like skin inflammation in mice

Female mice BALB/c (8–11 weeks) received a daily topical dose of 62.5 mg of commercially available IMQ cream (5%, Aldara; 3M Pharmaceuticals) on the dorsal and lumbar areas of the shaved back for 6 consecutive days, as described previously (van der Fits *et al.*, 2009). Similarly, a vehicle cream (Vaselina Pura, Laboratorios Rida, Valencia, Spain) was applied to the IMQ-untreated mice.

1 hour before the cream application, BTH (400 µg per site), dexamethasone (200 µg per site), or vehicle (acetone) were topically administered to a delimited area of 3 cm^2. At the end of the experiment, mice were killed by cervical dislocation and 1-cm^2 punch biopsies were taken from the treated dorsal skin and stored at −70 °C for subsequent homogenization or fixed in formalin for histological study carried out as described previously (van der Fits *et al.*, 2009). Detailed description can be found in Supplementary Materials online. Alternatively, mice were killed at day 3 in order to study STAT3 and NF-κB activation. To measure the severity of the disease, an objective scoring system was developed based on the clinical Psoriasis Area and Severity Index, as described previously (van der Fits *et al.*, 2009), and was performed blindly by an independent investigator.

Statistical analysis

The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons using the GraphPad Prism 4 software (GraphPad Software San Diego, CA). Significance was assumed at $P<0.05$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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SUPPLEMENTAL MATERIAL

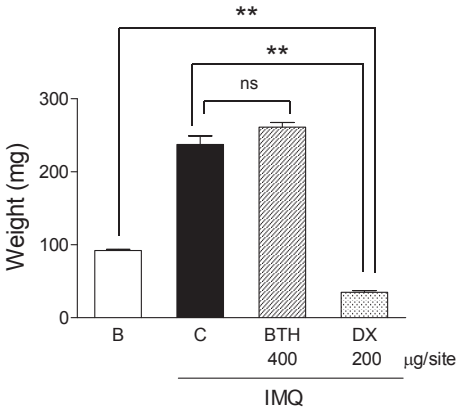

Figure S1. Effect of BTH and DX treatment on spleen mass in the IMQ-induced skin inflammation murine model. BTH, dexamethasone (DX) or vehicle (acetone) were topically applied 1 hour before IMQ administration for 6 days. Spleens were obtained at the end of the experiment and weighed. B: Vehicle-treated mice. C: Vehicle and IMQ-treated mice. Data represent mean ± S.E.M (n = 6 animals) * $p < 0.05$, ** $p < 0.01$.

MATERIALS AND METHODS

Electrophoretic Mobility Shift Assay (EMSA)

Normal human keratinocytes (NHK) were seeded on (6 cm diameter) petri dishes, preincubated with BTH or reference molecules for 30 minutes and then stimulated with TPA (1 µg/ml) for 1 hour. Nuclear extracts were prepared and EMSA was performed as described previously (Andres *et al.*, 2013). Complexes were analyzed by non-denaturing 6% polyacrylamide gel electrophoresis in 45 mM Tris-borate buffer (pH 8.3) containing 1 mM EDTA, followed by autoradiography of the dried gel using a Typhoon Imager-9400 (GE Healthcare, Wessling, Germany). To assess the specificity of the bands obtained, a 50-fold excess of unlabeled oligonucleotide was added to the reaction mixture.

Western blotting

Cell lysates were obtained and western blotting was performed as described previously (Andres *et al.*, 2013). Membranes were incubated with the specific antibody against pSTAT3 Tyr705 (Cell Signaling Technology, Beverly, MA). Primary antibody was detected by enhanced chemioluminescence using horseradish peroxidase conjugated secondary antibodies (Dako, Glostrup Denmark) and a standard ECL substrate (GE Healthcare, Wessling, Germany). Images were captured with the AutoChemi image analyzer (UVP Inc., Upland, CA). β-actin was used as a protein loading control.

Immunofluorescence analysis

Cultured primary human keratinocytes were fixed and permeabilized in prechilled absolute methanol for 15 min and rinsed with PBS. Cells were then incubated overnight at 4°C with polyclonal rabbit anti-STAT3 (Cell Signaling Technology, Beverly, MA). Secondary staining was obtained with Alexa Fluor® 488 secondary antibody (Invitrogen, Carlsbad, CA) for 1 hour at room temperature. Finally, samples were washed and nuclear staining was performed by

embedding samples in Prolong Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA). Samples were evaluated by epifluorescence microscopy with a Leica DM IL LED Fluo microscope (Leica Microsystems, Wetzlar, Germany). As a negative control, sections were incubated with PBS instead of primary antibody. Fluorescence quantification was performed with Image J (National Institutes of Health, Bethesda, Maryland).

Measurement of inflammatory mediators in skin homogenate

Mouse skin biopsies were frozen in liquid N_2 and homogenized. ELISA assays were performed to determine the concentration of IL-1β, TNFα (R&D Systems, Abingdon, UK), IL-6, IL-23 (eBioscience, San Diego, CA) and CXCL-1 (PromoCell, Heidelberg, Germany). Myeloperoxidase activity was assayed as described previously (Amigo *et al.*, 2007) and PGE_2 and LTB_4 were quantified by radioimmunoassay (Amigo *et al.*, 2007). NF-κB and STAT3 phosphorylation were measured by western blotting with specific antibodies against pSTAT3 (Tyr705) and pNF-κB p65 (Ser 536) from Cell Signaling Technology (Beverly, MA).

Histological study

Formalin-fixed paraffin-embedded tissue sections (7 μm thick) were stained with H&E and visualized with a Leica DM IL LED Fluo microscope (Leica Microsystems, Wetzlar, Germany). Quantification of the infiltrating cells was performed by two independent researchers on five random fields at a higher magnification (×400) per section for two sections per mouse and two mice per group. The results are expressed as the mean (± S.E.M) of cells counted per high power field (HPF). Epidermal thickness was measured using Leica Application Suite Software (Leica Microsystems, Wetzlar, Germany).

Immunohistochemical detection was carried out after antigen retrieval performed by boiling in citrate buffer (pH 6). Sections were incubated overnight at 4°C with rabbit anti-CK6 or rat anti-CD3 from Covance (Princeton, NJ) and R&D Systems (Abingdon, UK), respectively, as described previously (Blumberg *et al.*, 2010). Rabbit IgG antibody (Dako, Glostrup, Denmark)

was used as negative control. Secondary staining was obtained incubating with either Alexa Fluor® 488 Dye (Invitrogen, Carlsbad, CA) or swine anti-rabbit antibody (Dako, Glostrup, Denmark) followed by development with diaminobenzidine (Sigma-Aldrich, St Louis, MO) and counterstaining with haematoxylin for 1 min.

In the imiquimod model, keratinocyte proliferation was determined by BrdU incorporation. On the seventh day of the experiment, mice were injected with 1 mg of BrdU 2 hour before sacrifice as described previously (van der Fits *et al.*, 2009). Skin biopsies were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections were deparaffinized, boiled in citrate buffer (pH 6) and incubated with mouse-anti-BrdU (Dako, Glostrup, Denmark), followed by HRP-labeled goat-antimouse IgG (Sigma-Aldrich, St. Louis, MO). HRP activity was visualized using diaminobenzidine (Sigma-Aldrich, St Louis, MO) and sections were counterstained with haematoxylin for 1 min.

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Resultados y discusión

1. Profundización en los mecanismos fisiopatológicos de la psoriasis

1.1 Estudio de la vía Jak/STAT en queratinocitos

Desde el descubrimiento de su papel en la patogénesis de la psoriasis, la vía de las Jak/STAT ha suscitado gran interés como posible fuente de nuevas dianas terapéuticas. Pese al énfasis que se está haciendo en moléculas capaces de inhibir las Jak, poco se sabe del nivel de expresión de esta familia de proteínas en piel psoriásica. Por ello, en la presente tesis nos propusimos realizar un estudio de esta vía de señalización en queratinocitos primarios humanos con el fin de aportar nueva evidencia relevante para el desarrollo de nuevos fármacos. Así, el análisis de la expresión de las Jak en muestras de piel psoriásica lesional y no lesional reveló una disminución tanto a nivel de mRNA como de proteína para Jak1 y Jak2 así como a nivel de mRNA para Tyk2. En cambio, el mRNA de Jak3 aumentó (Artículo 1, figura 1). Puesto que Jak3 se encuentra únicamente en células del sistema inmune (Schindler *y cols.*, 2007), este incremento podría deberse al aumento en el infiltrado leucocitario característico de esta patología. La disminución del resto de miembros de esta familia podría estar relacionado con mecanismos compensadores, pero serán necesarios más estudios para confirmar esta hipótesis.

Nuestro siguiente objetivo fue la caracterización del nivel de activación final de esta vía en piel psoriásica a través del estudio de los factores de transcripción STAT1 y STAT3. En contraposición con la disminución de la expresión de las Jak, tanto la expresión a nivel de mRNA y de proteína, como la activación de STAT1 y STAT3 se vieron aumentadas en piel psoriásica lesional con respecto a la piel no lesional. Aún más, los ensayos de inmunofluorescencia con piel lesional mostraron un aumento de la fosforilación de STAT1 y STAT3 tanto en los residuos de tirosina como en los de serina, a lo largo de todas las capas epidérmicas (Artículo 1, figura 2 y artículo 2,

figura 1). Cabe recordar que STAT3 posee un efecto activador de la proliferación de los queratinocitos, mientras que STAT1 tiene un efecto inhibidor. Sin embargo, pese a la activación simultánea de estos dos factores de transcripción con efectos antagónicos en la epidermis psoriásica lesional, el fenotipo psoriásico parece indicar que, en el contexto de una placa psoriásica, predomina el efecto proliferativo del STAT3. Por este motivo este factor de transcripción fue estudiado como diana terapéutica en la segunda fase de la presente tesis.

El estudio de la vía de señalización de STAT3 en queratinocitos primarios humanos mostró que la IL-6 y la IL-20 inducen su fosforilación en el residuo de tirosina 705 (Tyr705) a través de Jak2 y que esta modificación produce una fuerte inducción de la actividad transcripcional de STAT3. La ausencia de inhibidores específicos de los otros miembros de la familia de las Jak disponibles a nivel comercial nos impidió identificar la segunda cinasa implicada en esta fosforilación en queratinocitos, posiblemente Jak1 (Murray, 2007). Paralelamente, se demostró que otros estímulos no específicos para esta vía, como son el TPA (12-*O*-tetradecanoilforbol-13-acetato), el TNFα o la radiación UVB, desencadenan la fosforilación de STAT3 en Ser727 y que esta modificación, pese a no tener un efecto inductor per se, modula la actividad transcripcional de STAT3 (Artículo 1, figuras 3 y 4). En concordancia con estudios previos, el efecto neto de esta modificación depende del estímulo inductor al que simultáneamente es sometida la célula (Revisado en Decker y Kovarik, 2000). Los resultados obtenidos muestran la complejidad de los sistemas intracelulares de transducción de señales en los que diferentes vías pueden interaccionar entre sí para modular la actividad de un factor de transcripción (Figura 17). De esta manera, la actividad transcripcional de STAT3 en los queratinocitos psoriásicos depende de la interacción entre diversos mediadores, poniendo en evidencia la compleja red de conexiones que mantienen las placas psoriásicas.

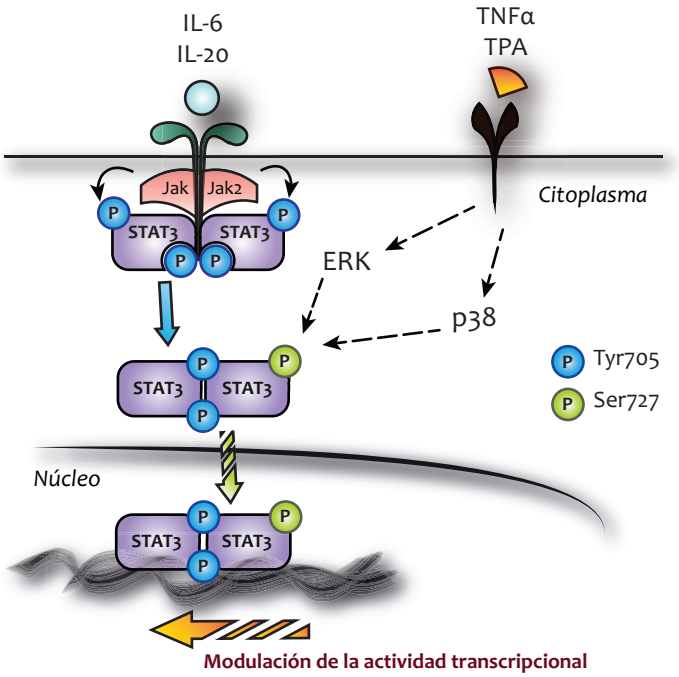


Figura 17. La actividad transcripcional neta de STAT3 depende de la intercomunicación entre diversas vías de transducción de señales.

Por otro lado, el IFNα y el IFNγ indujeron la actividad transcripcional de STAT1 a través de la fosforilación tanto en el residuo de tirosina como en el de serina (Artículo 2).

1.2 Estudio del papel de la adenosina en el proceso psoriásico

A pesar de la introducción de nuevas terapias biológicas, el agente inmunosupresor metotrexato todavía se utiliza en el tratamiento de la psoriasis de moderada a severa. Diversos estudios indican que el metotrexato promueve la acumulación extracelular de adenosina en los tejidos inflamados y que este mecanismo es esencial para su acción antiinflamatoria (Montesinos *y cols.*, 2000; Morabito *y cols.*, 1998; Cronstein *y cols.*, 1993). A pesar de ello, el papel de la adenosina en la piel psoriásica permanece todavía por discernir. Por ello, en la presente tesis se ha caracterizado el patrón de expresión de los receptores de adenosina en epidermis normal y psoriásica. De acuerdo con estudios previos en queratinocitos humanos (Brown *y cols.*, 2000), el análisis por PCR cuantitativa de las biopsias de piel confirmó la expresión mayoritaria del receptor A_{2B} tanto en epidermis normal como psoriásica. Además, también se detectaron niveles del receptor A_{2A}, aunque con una expresión menor, de manera similar a los resultados obtenidos en queratinocitos murinos (Braun *y cols.*, 2006). Destacablemente, se observaron diferencias en la expresión de estos dos subtipos de receptores entre epidermis normal y psoriásica, de manera que en la epidermis psoriásica se produce una disminución de la expresión de los receptores A_{2B} y un aumento de los A_{2A}. Los subtipos A_1 y A_3 no fueron detectados (Artículo 3, figura 1).

Numerosos estímulos proinflamatorios son capaces de modular la expresión de los receptores de adenosina (Haskó *y cols.*, 2009; Morello *y cols.*, 2006). En consecuencia, en nuestro grupo de investigación postulamos que el cambio en la expresión de los receptores de adenosina observado en piel psoriásica podría deberse al ambiente inflamatorio que se establece en la piel durante el curso de esta patología. En ensayos con queratinocitos humanos, demostramos que el IFNγ por sí solo y en menor medida el TPA, son capaces de reproducir el fenotipo psoriásico. Además, el TNFα y la IL-1β también aumentaron la expresión del receptor A_{2A} mientras que el IFNα disminuyó la expresión del A_{2B}. Otros mediadores importantes para la patología

psoriásica, como son la IL-6, la IL-17 y la IL-23, no produjeron ningún efecto (Artículo 3, figura 2). Es interesante destacar que todos los estímulos estudiados que modulan la expresión de los receptores de adenosina en queratinocitos humanos lo hacen en el mismo sentido en el que se ven alterados en la epidermis psoriásica, confirmando que los procesos inflamatorios tienen la capacidad de modificar la expresión de los receptores de adenosina produciendo una disminución del A_{2B} y un aumento del A_{2A} en este tipo celular.

El receptor A_{2B} es un receptor de baja afinidad cuya activación requiere de procesos patológicos en los que las concentraciones extracelulares de adenosina se ven fuertemente incrementadas. Además, se ha demostrado que la densidad de receptores en la superficie celular condiciona su capacidad de señalización (Haskó *y cols.*, 2009). Nuestros resultados sugieren que en la epidermis psoriásica la señalización a través del receptor de adenosina A_{2B} podría verse disminuida en favor del receptor A_{2A}. En este sentido, cabe destacar que el A_{2A} es un receptor de alta afinidad, por lo que el impacto del aumento de la expresión de este receptor puede ser incluso mayor que el de la disminución del A_{2B}. De hecho, el efecto A_{2A} parece eclipsar al A_{2B} en cuanto a capacidad antiinflamatoria (Hasko *y cols.*, 2008). De este modo, nuestros resultados sugieren que en el contexto de las placas psoriásicas se produce una potenciación de la respuesta A_{2A} con respecto a la A_{2B}. Dada la importancia del receptor A_{2A} en la acción antiinflamatoria de la adenosina (Ohta y Sitkovsky, 2001), se hace de especial interés el ahondamiento en las consecuencias fisiopatológicas de este fenómeno.

Los resultados preliminares de este estudio han identificado la vía del AMPc como la principal vía de señalización asociada a la activación del receptor A_{2B}. De este modo se ha completado, mediante el uso de agonistas selectivos, los resultados obtenidos por Brown *y cols.* (2000), en los que se estudió el efecto global de la adenosina. Así mismo, se ha descartado que el receptor A_{2A} actúe a este nivel (Artículo 3, figura 2). Estos resultados confirman el papel protector del receptor A_{2B} en queratinocitos humanos

ya que es conocido que el aumento del AMPc posee acciones antiinflamatorias en el tratamiento de la psoriasis (Schafer, 2012).

Por otro lado, se ha observado que agonistas selectivos y no selectivos del receptor A_{2A} son capaces de inhibir la liberación de IL-8 y TNFα en queratinocitos primarios humanos estimulados con TPA, sugiriendo el posible efecto protector de este receptor en la patología psoriásica (Artículo 3, figura 2).

La adenosina es un conocido regulador de la inflamación y de la inmunidad, que ejerce sus efectos mediante la interacción con uno o más de sus cuatro receptores de superficie específicos. Los resultados preliminares obtenidos en la presente tesis demuestran que durante el desarrollo de las lesiones psoriásicas se produce en los queratinocitos una alteración en el nivel de expresión de los receptores de adenosina que podría favorecer los efectos antiinflamatorios del receptor A_{2A}. En la actualidad, nuestro grupo continúa trabajando en este campo con el fin de clarificar las acciones de la adenosina en los procesos de proliferación y diferenciación de queratinocitos, así como esclarecer la contribución de la adenosina al efecto del metotrexato en la hiperplasia cutánea.

2. Búsqueda de nuevos agentes antipsoriásicos

2.1 Screening previo

En la presente tesis se ha abordado el estudio de una serie de moléculas de origen natural con el fin de determinar su posible interés como nuevos agentes antipsoriásicos. Para ello, en una fase previa, estas moléculas fueron ensayadas en la línea celular de macrófagos murinos RAW 264.7 con el fin de determinar su potencial efecto antiinflamatorio.

Las coscinolactamas A y B inhibieron de forma moderada la producción de PGE_2 y NO en macrófagos estimulados, presentando una concentración inhibidora 50 (CI_{50}) en el rango micromolar. La reducción de la generación de NO fue debida a la inhibición de la expresión de la NO sintasa inducible, enzima fuertemente inducida durante la mayoría de procesos de carácter inflamatorio (Artículo 4, tabla 3 y figura 2).

Asimismo, en esta línea celular se realizó el screening inicial de una serie de derivados semisintéticos de Petrosaspongiolida M, sesterterpeno aislado de la esponja marina *Petrosaspongia nigra* con potente actividad antiinflamatoria (Posadas *y cols.*, 2003). En los últimos años, nuestro grupo de investigación ha estudiado las propiedades antiinflamatorias de esta familia de moléculas y su relación estructura-actividad, obteniendo resultados muy prometedores. Estos derivados se caracterizan por poseer un núcleo γ-hidroxibutenólido (Figura 18) y por ejercer una potente inhibición de la síntesis de la PGE_2 a través de diversos mecanismos, como la inhibición de la fosfolipasa A_2 (PLA_2) o la disminución de la expresión de la prostaglandina E sintasa microsomal 1 (mPGEs-1) (Guerrero *y cols.*, 2007). Por ello, en este estudio, una nueva batería de derivados γ-hidroxibutenólido fueron examinados en cuanto a su capacidad para inhibir la liberación de PGE_2. De los 20 derivados ensayados, tan solo 3 (14g, 16g y 18) obtuvieron una potencia similar al cabeza de serie BTH (Artículo 5, Tabla 1,

figura 3). Estos resultados, junto con los datos obtenidos en estudios previos (Guerrero *y cols.*, 2009; Guerrero *y cols.*, 2007), nos llevaron a seleccionar el derivado BTH para su estudio en profundidad (Figura 18).

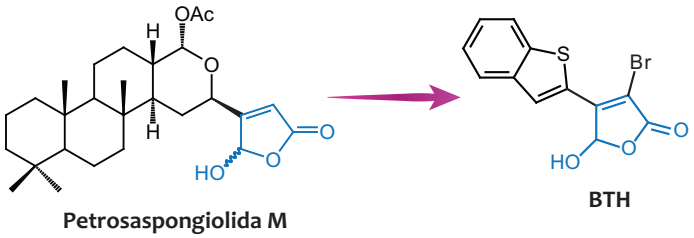


Figura 18. Estructuras químicas de Petrosaspongiolida M y BTH. En azul se muestra el núcleo γ-hidroxibutenólido característico de esta familia de compuestos.

En una segunda fase, aquellos derivados que resultaron de mayor interés fueron evaluados en queratinocitos primarios humanos. De este modo, se confirmó el efecto inhibidor de las pertamidas, el condroitín sulfato y el BTH sobre la liberación de TNFα e IL-8, determinados como mediadores característicos de la respuesta inflamatoria en este tipo celular. Pese a los prometedores resultados obtenidos con las pertamidas (Artículo 6, figura 6), únicamente el BTH y el CS fueron seleccionados para realizar el estudio en profundidad de la actividad antipsoriásica (Figura 19). Las ventajas de estas dos moléculas residían, por un lado, en la amplia experiencia clínica existente con el condroitín sulfato tras años de uso en la terapéutica de la osteoartritis y, por otro, en la mayor potencia y sencillez estructural del BTH frente a las pertamidas. Además, los resultados previos obtenidos con esta molécula, demostraban un interesante perfil farmacológico tanto *in vitro* como *in vivo* basado en la capacidad de inhibir la activación del NF-κB (Guerrero *y cols.*, 2009; Guerrero *y cols.*, 2007).

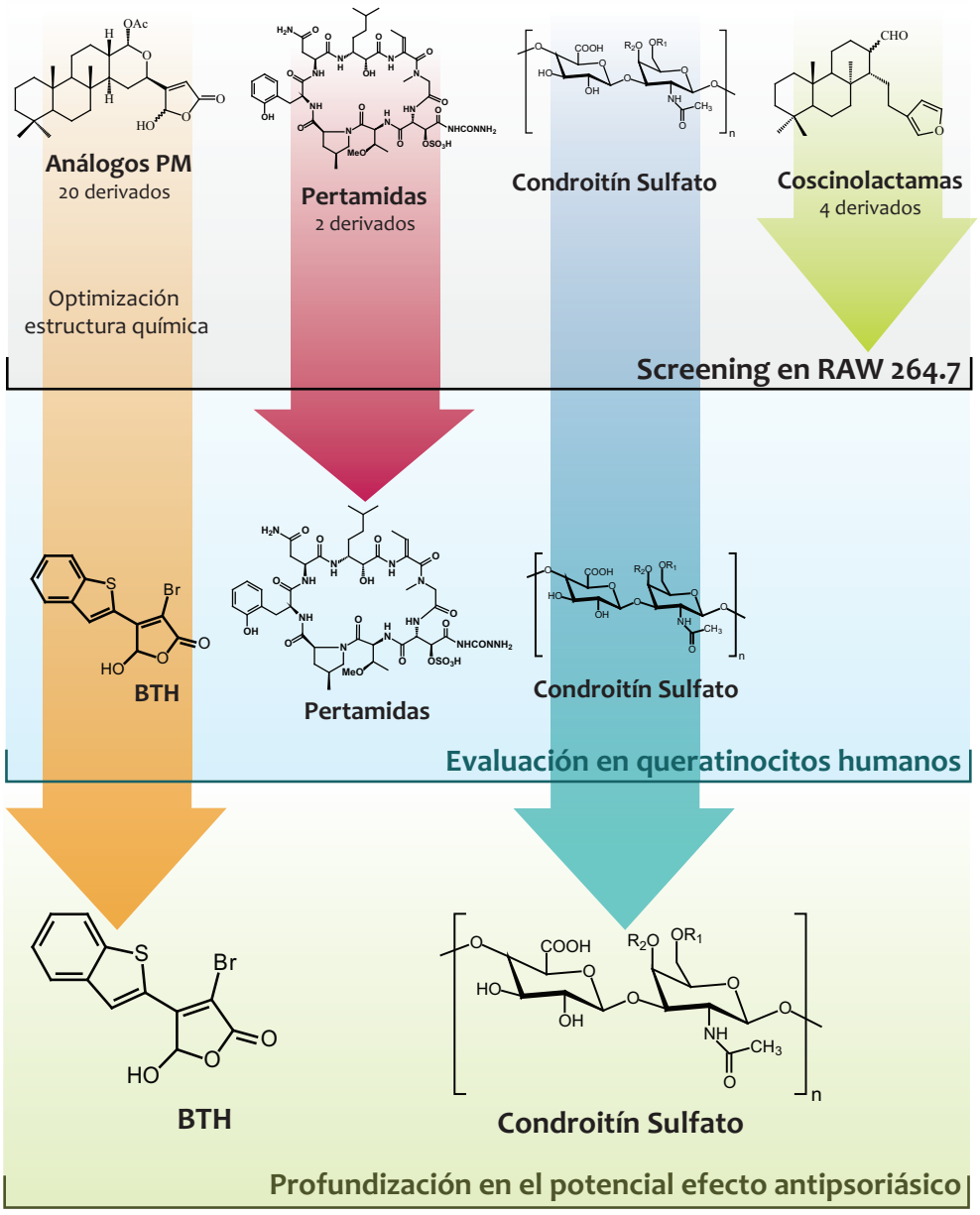


Figura 19. En la primera fase de la búsqueda de nuevos fármacos antipsoriásicos, los compuestos candidatos fueron sometidos a un proceso de cribado en base a su potencial actividad antiinflamatoria, primero en líneas celulares de macrófagos y después en cultivos primarios de queratinocitos. Este proceso dio lugar a la selección del condroitín sulfato y el BTH para su estudio en profundidad.

2.2 Estudio del efecto antipsoriásico del condroitín sulfato

El CS es un glicosaminoglicano presente en la matriz extracelular de diversos tejidos, incluida la piel, donde forma parte de los proteoglicanos. Está compuesto por la unión 1-3 del ácido D-glucurónico y la N-acetilgalactosamina formando disacáridos que se unen entre sí por uniones galactosamina β 1-4. Los residuos de galactosamina pueden estar sulfatados en la posición 4 o 6, dando lugar al condroitín-4-sulfato y el condroitín-6-sulfato (Figura 20).

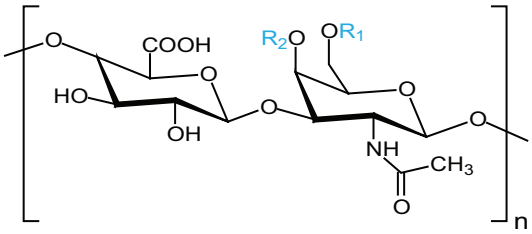


Figura 20. Estructura química de una unidad de cadena de condroitín sulfato. En azul se resaltan las posiciones susceptibles de ser sulfatadas. Condroitín-4-sulfato: R1 = H; R2 = SO3H. Condroitín-6-sulfato: R1 = SO3H; R2 = H.

Al tratarse de un componente de tejidos animales, el CS se obtiene mayoritariamente de la digestión proteolítica de cartílago de origen bovino, porcino o marino, de los que se obtienen moléculas de composición heterogénea en función de la fuente primaria de la que son aislados. Además, en algunos países estas moléculas son comercializadas como complementos alimenticios, lo que las exime de los controles de pureza y efectividad necesarios para la aprobación de nuevos fármacos (Uebelhart, 2008). Por ello, existe gran disparidad en los resultados de ensayos clínicos realizados en diferentes países y con preparados diferentes (Schneider *y cols.*, 2012; Hochberg, 2010; Wandel *y cols.*, 2010; Clegg *y cols.*, 2006). El hecho de que todas estas preparaciones sean comercializadas bajo el nombre de condroitín sulfato ha dado lugar a una reciente controversia sobre la efectividad de este compuesto. En el debate generado se

ha puesto de manifiesto la importancia de conocer el origen de los ingredientes activos de la preparación prescrita como el factor más importante para asegurar la calidad y, por lo tanto, la eficacia, de los fármacos utilizados (Schneider *y cols.*, 2012; Uebelhart, 2008). El CS empleado en la presente tesis fue suministrado por la empresa Bioibérica S.A. y se trata de una mezcla altamente purificada de condroitín 4 y 6 sulfato de origen bovino en una concentración superior al 98%, con un peso molecular medio de 15 - 16 kDa y un ratio entre los sulfatos en posición 4 y 6 de 2. Las propiedades antiinflamatorias de este preparado han sido demostradas tanto en estudios *in vitro* como en ensayo clínicos (Canas *y cols.*, 2010; Moller *y cols.*, 2010; Jomphe *y cols.*, 2008; Uebelhart *y cols.*, 2004).

A pesar de toda esta controversia, el CS ha sido empleado durante muchos años en el tratamiento de la osteoartritis y es considerado por la Liga Europea frente al Reumatismo (EULAR de las siglas en inglés) como uno de los tratamientos más seguros para esta patología (Jordan *y cols.*, 2003). Esta ventaja terapéutica, unida a los datos preliminares publicados sobre una posible utilidad en el tratamiento de la psoriasis basadas en la observación clínica (Moller *y cols.*, 2010; Verges *y cols.*, 2005), nos llevaron a abordar el estudio de su efecto en queratinocitos primarios humanos.

Además de inhibir la liberación de TNFα e IL-8, el pretratamiento con CS (200 μg/ml) disminuyó la producción de IL-6 y la quimiocina específica de queratinocitos CCL27. La expresión de todas estas citocinas está mediada por el NF-κB, factor de transcripción central en la fisiopatología de la psoriasis (Figura 12). Por ello, se realizó un ensayo de movilidad electroforética retardada (EMSA), que confirmó la capacidad del CS para inhibir la activación del NF-κB en queratinocitos, tal y como ya había sido demostrado en otros tipos celulares (du Souich *y cols.*, 2009). Así pues, el CS reduce la producción de citocinas proinflamatorias en queratinocitos a través de la inhibición de la activación del NF-κB, confirmando su potencial interés terapéutico en el tratamiento de la psoriasis (Artículo 7, figuras 1-2).

Adicionalmente, y dada la importancia del STAT3 para la patogénesis de la psoriasis, se evaluó el efecto del CS a nivel de la activación de este factor nuclear. En este sentido, tanto los ensayos de inmunofluorescencia como de western blot de las fracciones citosólica y nuclear mostraron la capacidad del CS de inhibir la translocación al núcleo de STAT3. Este efecto inhibidor se confirmó mediante un ensayo de luciferasa con el promotor de STAT3, que mostró una disminución significativa de la actividad transcripcional de este factor nuclear tras el pretratamiento con CS de queratinocitos humanos HaCaT (Artículo 7, figura 3). Cabe destacar que este efecto es independiente de la fosforilación en Tyr705, lo que indica que el CS podría estar actuando a otro nivel, como puede ser a través del bloqueo de las importinas o el aumento de la actividad de las fosfatasas o los SOCS (Figura 14).

En conclusión, nuestro estudio ha aportado nuevas evidencias de la potencial utilidad del CS para el tratamiento de la psoriasis. La capacidad de disminuir dos de las vías de señalización claves para el desarrollo de esta enfermedad, como son la vía del NF-κB y del STAT3, en queratinocitos primarios humanos (Figura 21) avala los datos preliminares que existen acerca de su efectividad en clínica. Dado su perfil terapéutico en el tratamiento de la osteoartritis, sería de esperar que esta molécula pudiera aportar beneficios a aquellos pacientes aquejados de una patología leve, en los que el buen perfil de seguridad sería su mayor ventaja frente a otros tratamientos. Los resultados obtenidos en la presente tesis sientan una base para el desarrollo de nuevos ensayos clínicos con el fin de discernir el potencial interés del CS en la terapéutica de la psoriasis.

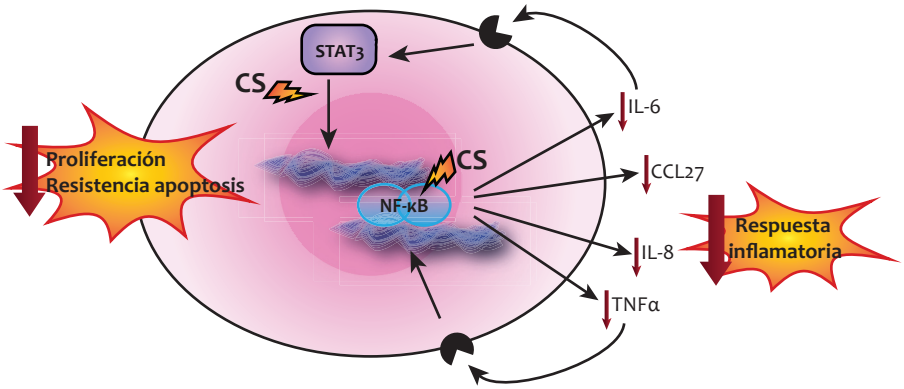


Figura 21. Gracias a su efecto inhibidor sobre las vías del NF-κB y el STAT3 en queratinocitos primarios humanos, el CS podría representar una nueva alternativa terapéutica para el tratamiento de las formas leves de psoriasis.

2.3 Estudio del efecto antipsoriásico del BTH

Como se ha comentado anteriormente, nuestro grupo de investigación lleva varios años estudiando las propiedades antiinflamatorias de Petrosaspongiolida M y trabajando en la optimización de su estructura química mediante el estudio de derivados semisintéticos. De este modo, tras la caracterización de su mecanismo de acción, mediado en parte por la inhibición del NF-κB (Posadas *y cols.*, 2003), se trató de obtener derivados de mayor disponibilidad y perfil farmacológico más favorable (Guerrero *y cols.*, 2007), formando parte de este proceso algunos resultados que se describen en la presente tesis (Artículo 5). De todos ellos, el BTH (Figura 18) fue el que mostró un perfil más interesante, ya que mantuvo la capacidad de inhibir la activación del NF-κB *in vitro* (Guerrero *y cols.*, 2007) y demostró un potente efecto antiinflamatorio y analgésico en los modelos de hiperalgesia inducida por ácido acético, bolsa de aire y artritis inducida por colágeno en ratón (Guerrero *y cols.*, 2009). Por todo ello, fue seleccionado para evaluar su potencial efecto antipsoriásico tras el screening preliminar en queratinocitos humanos.

Nuestro primer objetivo fue confirmar, mediante un ensayo de EMSA, el efecto inhibidor de BTH (1-10 μM) sobre la activación del NF-κB en queratinocitos humanos. Esta inhibición fue acompañada de una reducción significativa de citocinas proinflamatorias dependientes de la activación de este factor nuclear como IL-6 y CCL27, además de IL-8 y el TNFα (Artículo 8, figura 1).

Dado el creciente interés de la vía Jak/STAT3 en la hiperproliferación de los queratinocitos durante el desarrollo de la psoriasis, evaluamos el efecto del BTH sobre la activación de esta vía de señalización. Los estudios por western blot e inmunofluorescencia realizados tras el estímulo con IL-6, demostraron la capacidad de BTH para disminuir la translocación al núcleo de STAT3 a través del bloqueo de su fosforilación en Tyr705. Teniendo en cuenta los resultados previamente obtenidos en la presente tesis, según los cuales Jak2 juega un papel fundamental en la fosforilación de STAT3 tras el estímulo de IL-6, podemos aventurar que el BTH podría comportarse como un inhibidor de Jak2. Sin embargo, no es posible aseverar su selectividad con respecto a otros miembros de la familia de las Jak, dada la promiscuidad con la que los receptores de citocinas se conjugan con este tipo de cinasas. Finalmente, es importante destacar que este efecto se tradujo en una inhibición de la proliferación de los queratinocitos, visible tras 48 horas de incubación con BTH (Artículo 8, figura 2).

Los ensayos *in vitro* nos ayudan a discernir el mecanismo de acción de nuevos fármacos y a evaluar su posible interés. Sin embargo, cualquier nueva molécula que aspire a ser utilizada en la terapéutica humana ha de ser testada previamente en animales de experimentación. Los modelos animales nos permiten analizar el efecto de estas moléculas cuando son sometidas a las complejas interacciones que se dan en un organismo vivo, con el fin de evaluar su eficacia y seguridad. En nuestro caso, estos estudios nos permitieron, además, evaluar la idoneidad de este compuesto para ser

administrado por vía tópica, lo cual supone una ventaja terapéutica en las patologías de la piel.

De este modo, la capacidad antipsoriásica del BTH fue ensayada en dos modelos animales: la hiperplasia en piel de ratón inducida por TPA y la inflamación psoriásica inducida por imiquimod (IMQ).

El modelo de hiperplasia en piel de ratón inducida por TPA fue seleccionado por tratarse de un modelo de inflamación cutánea aguda de uso extendido para la evaluación de nuevos fármacos antiinflamatorios por su sencillez y bajo coste. Este modelo nos permitió confirmar los buenos resultados obtenidos con el BTH *in vitro*, al prevenir la formación de las lesiones cutáneas tras la aplicación del TPA en los ratones. En este sentido, el estudio histológico de la piel tratada con BTH mostró una normalización de la hiperplasia epidérmica y la infiltración leucocitaria que se correlacionó con una disminución de los niveles de citocinas y eicosanoides en los homogeneizados de piel. De esta manera, los niveles de leucotrieno B_4 (LTB_4), PGE_2, TNFα, quimiocina (C-X-C) ligando 1 (CXCL-1, el equivalente murino a la IL-8), IL-6 e IL-1β, así como el edema, fueron significativamente menores en los ratones tratados con BTH que en los ratones control. Finalmente, se confirmó *in vivo* el mecanismo de acción del BTH, mediante el estudio por western blot de la inhibición de la fosforilación de NF-κB y el STAT3 en la piel (Artículo 8, figuras 3-4).

Adicionalmente y con el fin de confirmar el potencial efecto antipsoriásico del BTH, se puso a punto en nuestro laboratorio el modelo de inflamación psoriásica inducida por IMQ. Este modelo, de base inmunológica, se ha convertido en los últimos años en el referente para el estudio *in vivo* de nuevos agentes antipsoriásicos. La aplicación repetida de IMQ, un agonista de los receptores TLR7 y 8, durante 6 días consecutivos en la piel de los ratones genera un fenotipo que comparte muchas características con la patología psoriásica y que está mediado por la IL-22 e IL-23 (Van Belle *y cols.*, 2012;

van der Fits *y cols.*, 2009). Una vez más, la aplicación tópica del BTH resultó efectiva para la prevención de la aparición de las lesiones, disminuyendo la descamación y el eritema, así como la hiperplasia epidérmica, el infiltrado leucocitario y la liberación del IL-23 (Artículo 8, figura 5). Cabe destacar que esta mejoría se dio únicamente en el área de piel tratada con BTH, sin afectar a las zonas adyacentes y sugiriendo, por lo tanto, un efecto local.

En este sentido, el carácter inmunológico sistémico de este modelo experimental nos proporcionó nuevos datos que avalan las potenciales ventajas terapéuticas del BTH. La aplicación repetida de imiquimod en la piel produce una fuerte estimulación del sistema inmune de los ratones que se traduce en una hiperplasia del bazo, medible al final del experimento (van der Fits *y cols.*, 2009). Tras su administración tópica, el BTH, pese a prevenir la patología cutánea, no afectó a la hiperplasia del bazo, confirmando que su efecto beneficioso se desarrolló de manera local. Sin embargo, la dexametasona, administrada como fármaco de referencia, sí que produjo una disminución significativa del peso de los bazos, llegando a ser incluso menor que el del grupo de los ratones blanco y sugiriendo una absorción sistémica así como una profunda inmunosupresión. Además, el principal efecto adverso del uso de corticoides tópicos, la atrofia cutánea, se manifestó en toda la superficie de la espalda de los ratones, más allá de la zona tratada (Artículo 8, figuras 5 y S1).

El uso de corticoides supone el principal tratamiento tópico para la psoriasis leve localizada. Sin embargo, la aparición de reacciones adversas y tolerancia limita su uso a largo plazo y hace de especial importancia la búsqueda de nuevos fármacos capaces de controlar los síntomas de las lesiones con un mejor perfil de seguridad. En la presente tesis se ha estudiado el efecto antipsoriásico del BTH, mediado por la inhibición de los factores de transcripción NF-κB y STAT3 (Figura 22). Los buenos resultados obtenidos subrayan el potencial de esta molécula y respaldan el interés de futuros estudios que determinen su posible aplicación en la terapéutica de esta enfermedad.

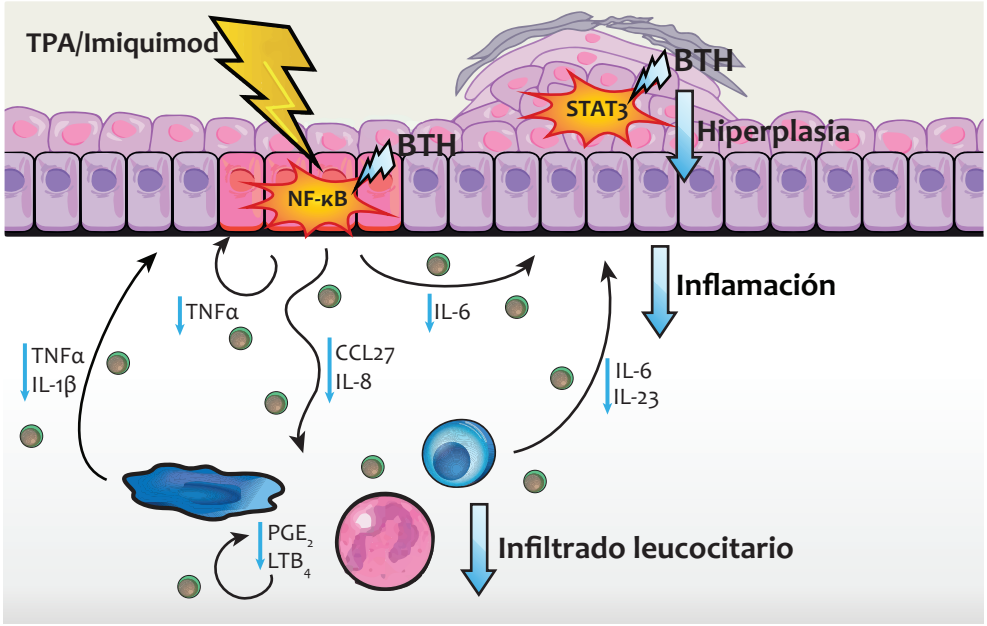


Figura 22. El tratamiento con BTH inhibe las vías de señalización de NF-κB y STAT3 en la piel, reduciendo la inflamación, el infiltrado leucocitario y la hiperplasia epidérmica, lo que conduce a la normalización del fenotipo psoriásico.

En conclusión, la psoriasis es una patología heterogénea cuyo fenotipo resulta de la interacción de factores tanto genéticos como ambientales. Sin embargo, existen una serie de mecanismos efectores comunes que son los encargados de reproducir la enfermedad la mayoría de los pacientes. Los rasgos más característicos de una placa psoriásica son el infiltrado leucocitario, atraído por la inflamación local, y la hiperplasia epidérmica, debida a un incremento en la tasa de división de los queratinocitos y a una diferenciación aberrante. Por ello, el bloqueo de los factores de transcripción encargados de la dirección de las respuestas implicadas en la patogénesis de la psoriasis constituye una prometedora estrategia terapéutica, poco explorada todavía. En la presente tesis se han estudiado nuevas moléculas que interfieren con las vías de señalización del NF-κB, principal responsable de la respuesta inflamatoria, y el STAT3, mediador de la acantosis epidérmica y de la diferenciación de los linfocitos

T_H17, confirmando la eficacia de este abordaje terapéutico tanto en cultivos de queratinocitos humanos primarios como en modelos animales de psoriasis. El bloqueo de dos de los principales efectores del proceso psoriásico se traduce en la disminución de una gran variedad de mediadores, necesarios no sólo para el desencadenamiento sino también para el mantenimiento de las lesiones psoriásicas. En este sentido, el efecto pleotrópico de estas moléculas puede suponer una ventaja adicional frente a las terapias biológicas dirigidas contra un mediador en concreto. Además, el excelente perfil de seguridad del CS y la posibilidad de aplicación tópica del BTH convierten a estas moléculas en candidatos privilegiados para el desarrollo de nuevas terapias aplicables a las formas leves de la psoriasis. Futuros estudios serán necesarios para confirmar la eficacia de la estrategia terapéutica postulada en la presente tesis y la utilidad del BTH y el CS como nuevos agentes antipsoriásicos.

Conclusiones

1. La expresión y la activación de STAT1 y STAT3 se encuentran elevadas en la piel psoriásica lesional con respecto a la no lesional. Sin embargo, y en términos generales, se observa una disminución de la expresión de las Jak.

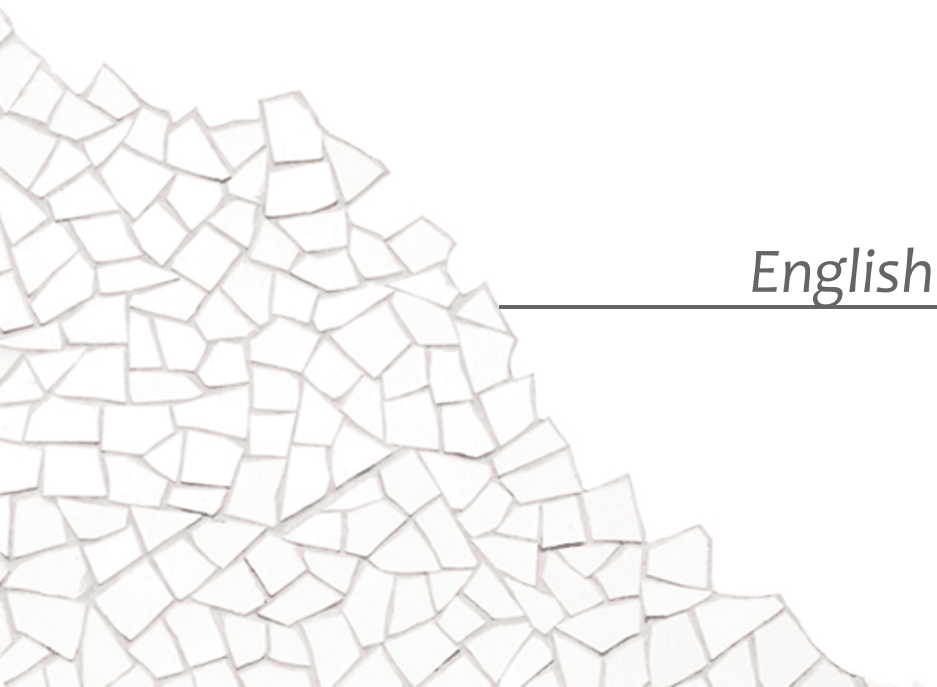
2. En cultivos de queratinocitos humanos, la fosforilación de STAT3 en Tyr705 induce una potente activación transcripcional, mientras que la fosforilación en Ser727 tiene un efecto principalmente modulador.

3. En la epidermis psoriásica se produce un aumento de los receptores de adenosina A_{2A} a la vez que una disminución de los receptores A_{2B} con respecto a la epidermis sana. Esta modificación parece estar relacionada con los mediadores proinflamatorios presentes en las placas psoriásicas.

4. El estudio de moléculas de origen marino y sus derivados semisintéticos constituye una fuente de gran interés para la obtención de nuevas estructuras potencialmente antiinflamatorias, como las coscinolactamas, las pertamidas y los derivados γ-hidroxibutenólido.

5. El condroitín sulfato reduce la generación de citocinas proinflamatorias en queratinocitos humanos a través de la inactivación del NF-κB. Además, bloquea la translocación nuclear de STAT3 por un mecanismo independiente de la fosforilación en Tyr705. Estos resultados sugieren un potencial interés del condroitín sulfato en el tratamiento de la psoriasis.

6. El derivado γ-hidroxibutenólido BTH, presenta un interesante perfil farmacológico como posible agente antipsoriásico en base a su capacidad de inhibir la activación de NF-κB y STAT3 tanto en queratinocitos humanos como tras su aplicación tópica en modelos animales de psoriasis, donde previene la hiperplasia epidérmica, la inflamación y el infiltrado leucocitario.



English version

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Abstract

Psoriasis is a chronic inflammatory skin disorder characterized by inflammation in dermis and epidermis, keratinocyte hyperproliferation, leukocyte infiltration and dilatation and growth of blood vessels. Immune-mediated inflammatory processes, involving both innate and adaptive immunity effector mechanisms, drive this pathology. The primary goal of the present work has been to further evaluate the contribution of epidermal cells to the pathogenesis of psoriasis, in order to provide new relevant evidence for the development of new therapies. In this regard, we have demonstrated that the inflammatory mediators found in psoriatic plaques are able to alter the expression of adenosine receptors, which are thought to play a pivotal role in the anti-inflammatory effect of methotrexate. Likewise, we have demonstrated that signal transducers and activators of transcription (STAT) 1 and 3 signaling pathways are upregulated in lesional psoriatic skin compared to non-lesional psoriatic skin, even though there is a decrease in Janus kinases (Jak) expression. Furthermore, we have characterized the role of several pro-inflammatory mediators in the modulation of these signaling pathways.

Given the importance of the nuclear factor κB (NF-κB) and STAT3 in psoriasis pathogenesis, we have searched for new molecules of natural origin able to diminish the signaling of these two transcriptional factors in the search of new agents suitable for the treatment of the mildest forms of the disease. For this purpose, we carried out a preliminary study in the murine macrophages cell line RAW 264.7 of a series of semisynthetic derivatives of natural molecules isolated from sea sponges, as well as with chondroitin sulfate (CS), a proteoglycan widely used for the treatment of osteoarthritis. In a second stage, the ability of these molecules to inhibit tumor necrosis factor α (TNFα) and interleukin (IL)-8 release in cultures of normal human keratinocytes was assessed. These preliminary studies led us to the selection of the semisynthetic derivative 4-benzo[*b*]thiophen-2-yl-3-bromo-5-hydroxy-5*H*-furan-2-one (BTH) and CS to further study their antipsoriatic potential.

Chondroitin sulfate pretreatment of normal human keratinocytes (NHK) inhibited NF-κB activation and the release of some of the key psoriatic cytokines such as TNFα, IL-8, IL-6 and chemokine (C-C motif) ligand (CCL)-27. Moreover, it impaired STAT3 translocation to the nucleus and significantly reduced STAT3 transcriptional activity, confirming its interesting profile as a candidate for future drug research in the therapeutics of psoriasis.

In a parallel study, BTH also inhibited the release of some of the key psoriatic cytokines through downregulation of NF-κB in normal human keratinocytes. Furthermore, it impaired STAT3 phosphorylation and translocation to the nucleus, which resulted in decreased keratinocyte proliferation. These results were confirmed *in vivo* in two murine models of psoriasis: the epidermal hyperplasia induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and the imiquimod (IMQ)-induced skin inflammation model. In both cases, topical administration of BTH prevented leukocyte infiltration and epidermal hyperplasia through suppression of NF-κB and STAT3 phosphorylation. Our results confirm the potential of BTH as a candidate for future drug research.

Globally, data obtained in this Thesis have shed light on the role of adenosine, Jak/STAT and NF-κB signaling pathways in psoriasis pathogenesis, as well as on the efficacy of small molecules of natural origin to ameliorate its course, suggesting the potential interest of these strategies in the development of new therapeutic approaches for this skin condition.

Outline of the thesis

Inflammatory skin diseases have a significant impact on the quality-of-life of patients and their families and represent an enormous economic burden for developed societies. It is estimated that approximately 2 - 3% of the population worldwide suffers psoriasis, accounting for around 25 million people in North America and Europe (Lowes *et al.*, 2007). The course of this skin condition is characterized by an imbalance between the local immune response and its downregulatory mechanisms, an exaggerated inflammatory response and an aberrant epidermal turnover and differentiation.

A huge amount of public and private resources have been devoted to improve the therapeutics of this skin disease, allowing the effective treatment of the more severe cases. This big effort has given rise to five new biological agents in the last decade, and three more that currently are in the last phases of clinical trials (Figure 9)[1]. However, this new biologic therapies cannot fulfill the needs of those patients whose pathology is not severe enough to warrant their use. The high economic cost, the parenteral administration and the risk of severe toxicity limit their use in the cases of low-to-moderate psoriasis, which make up 90% of the psoriatic patients. In this sense, the routine prescriptions have stayed invariable for 20 years and are still insufficient to provide effective and well-tolerated therapies. Thus, the search of new molecules suitable for the treatment of the mildest forms of this skin condition by either topical or oral administration is an area of special interest in the actual pharmacotherapy of psoriasis.

Therefore, the aim of the present work has been to:

1) Expand our knowledge of the pathophysiological response of the keratinocytes during the psoriatic process in order to recognize new therapeutic targets.
2) Search for new active molecules suitable for the treatment of this disease.

[1]Figure legends are explained from page 205 to 208

Part 1. Study of the molecular mechanisms underlying psoriasis

Despite the recent focus on Jak inhibitors as new agents for the treatment of psoriasis, little is known about STAT activation in the psoriatic epidermis. Therefore, during my stay in the laboratory of Prof. Knud Kragballe, in the Department of Dermatology from Aarhus University Hospital in Denmark, we analyzed the expression and activation of STAT1 and STAT3 in lesional and non-lesional psoriatic skin and we investigated the molecular mechanisms that modulate its activity in normal human keratinocytes. The results from these studies are reflected in the following articles:

Article 1

Studies of Jak/STAT3 expression and signalling in psoriasis identifies STAT3-Ser727 phosphorylation as a modulator of transcriptional activity.
Andres RM, Hald A, Johansen C, Kragballe K, Iversen L. (2013) ***Experimental Dermatology*** 22, 323-8.

Article 2

STAT1 expression and activation is increased in lesional psoriatic skin.
Hald A, Andres RM, Salskov-Iversen ML, Kjellerup RB, Iversen L, Johansen C. (2013) ***British Journal of Dermatology*** 168, 302-10.

Similarly, even though methotrexate has been used for over 50 years for the treatment of psoriasis, there is little evidence about the effect of adenosine in NHK. It has been reported that methotrexate promotes extracellular adenosine accumulation at sites of inflammation and that this mechanism is essential for its effect in inflammatory diseases (Cronstein *et al.*, 1993; Montesinos *et al.*, 2000; Morabito *et al.*, 1998). Adenosine (Figure 16) is a known regulator of inflammation and immunity via interaction with one or more of four known cell-surface receptors, but its effects on inflammatory skin diseases remain unclear. Our studies have addressed the expression of adenosine receptors in normal and psoriatic skin as well as the signaling pathways associated to their activation.

Article 3

Adenosine receptor expression is altered in psoriatic skin.
Andrés RM, Arasa J, Payá M, Navalón P, Valcuende F, Terencio MC, Montesinos MC. ***Letter to the Editor. Journal of Investigative Dermatology*** (Manuscript in preparation).

Part 2. Search of new antipsoriatic agents

The second aim of the present work has been to search for new molecules with anti-inflammatory and antipsoriatic potential. In this sense, our research group has a wide experience in the study of the anti-inflammatory properties of new molecules of natural origin. The interest of bioactive natural products lies in their evolutionary selection and validation for interfering with biological targets. Hence, libraries designed and synthesized around the basic structure of such compounds have a good chance of displaying biological and pharmacological properties.

We started our study by performing a pharmacological screening in the cell line RAW 264.7 in order to select the most interesting molecules. In this sense, the following compounds were evaluated:

- Coscinolactams A and B isolated from the sea sponge *Coscinoderma mathewsi* by the laboratory of Dr. Maria Valeria D'Auria in the Department of Chemistry of the Natural Substances from the University of Naples, Italy.
- Perthamides C and E isolated from the sea sponge *Theonella swinhoei* by the laboratory of Dr. Maria Valeria D'Auria in the Department of Chemistry of the Natural Substances from the University of Naples, Italy.
- Semisynthetic derivatives of Petrosaspongiolide M, sesterterpene isolated from the marine sponge *Petrosaspongia nigra.* These derivatives were synthesized by the group of Dr. Ines Bruno in the Department of Pharmaceutical Chemistry from the University of Salerno in Italy.
- Chondroitin sulfate, proteoglycan of animal origin widely used for the treatment of osteoarthritis, supplied by the private company Bioibérica S.A.

In the initial stages of this kind of studies, the easy to grow and widely available cell lines represent an advantageous strategy when working with a high number of molecules. In this regard, the murine macrophages cell line RAW 264.7 was chosen because of its broad and well-characterized inflammatory response to evaluate the capability of our compounds to inhibit general inflammatory mediators widely produced by macrophages, such as nitric oxide (NO) and prostaglandin E_2 (PGE_2). These preliminary studies gave rise to the following articles:

Article 4

Coscinolactams A and B: new nitrogen-containing sesterterpenoids from the marine sponge Coscinoderma mathewsi exerting anti-inflammatory properties.
De Marino S, Festa C, D'Auria MV, Bourguet-Kondracki M-L, Petek S, Debitus C, Andrés RM, Terencio MC, Payá M, Zampella A. (2009) ***Tetrahedron*** 65, 2905-9.

Article 5

Toward the Discovery of New Agents Able to Inhibit the Expression of Microsomal Prostaglandin E Synthase-1 Enzyme as Promising Tools in Drug Development.
De Simone R, Andrés RM, Aquino M, Bruno I, Guerrero MD, Terencio MC, Paya M, Riccio R. (2010) ***Chemical Biology & Drug Design*** 76, 17-24.

In a second stage, those molecules showing a more interesting profile were evaluated in NHK. Their ability to diminish TNFα and IL-8 production was assessed as an indicator of potential anti-inflammatory capability in this cell type, as these are two of the main cytokines produced by keratinocytes involved in the psoriatic process. Despite the good results obtained with all the tested molecules, we decided to proceed only with CS and the Petrosaspongiolide M derivative BTH, leading compound of the molecules assayed in article 5. Thus, we then studied the anti-inflammatory effect of these two agents on a wide range of mediators and transcriptional factors involved in psoriasis pathogenesis, demonstrating their potential as new antipsoriatic drugs. The results of these studies are shown in the following articles:

Article 6

Perthamides C–F, potent human antipsoriatic cyclopeptides.
Festa C, De Marino S, Sepe V, D'Auria MV, Bifulco G, Andrés R, Terencio MC, Payá M, Debitus C, Zampella A. (2011) ***Tetrahedron*** 67, 7780-6.

Article 7

Potential antipsoriatic effect of chondroitin sulfate through inhibition of NF-kappaB and STAT3 in human keratinocytes.
Andres RM, Paya M, Montesinos MC, Ubeda A, Navalon P, Herrero M, Verges J, Terencio MC. (2013) ***Pharmacological Research*** 70, 20-6.

Article 8

NF-κB and STAT3 inhibition as therapeutic strategy in psoriasis: in vitro and in vivo effect of BTH.
Andrés RM, Montesinos MC, Navalón P, Payá M, Terencio MC. (2013) ***Journal of Investigative Dermatology*** (In press, doi: 10.1038/jid.2013.182).

Results and discussion

Part 1. Study of the molecular mechanisms underlying psoriasis

1.1 Study of the Jak/STAT signaling pathway in human keratinocytes

Since the discovery of its role in psoriasis pathogenesis, Jak/STAT signaling pathway has attracted much attention as a possible target for new therapeutic agents. Despite the emphasis put on new molecules capable of diminishing Jak activation, little is known about the expression of this family of proteins in psoriatic skin. Therefore, we decided to study this signaling pathway in order to provide new relevant evidence for the development of new antipsoriatic agents. Thus, the analysis of Jak proteins expression in lesional and non-lesional psoriatic skin revealed a decrease of the mRNA and the protein levels of Jak1 and Jak2 as well as the mRNA of tyrosine kinase 2 (Tyk2). In contrast, Jak3 mRNA expression was increased (Article 1, figure 1). As Jak3 is expressed exclusively in immune cells (Schindler *et al.*, 2007), the higher levels of Jak3 mRNA could be due to the increased leukocytic infiltrate characteristic of this skin pathology. The decreased levels of the other members of the Jak family could be related to compensatory mechanisms. However, further studies will be needed in order to confirm this hypothesis.

Our next goal was to characterize the total activation level of this signaling pathway in psoriatic skin through the study of the transcriptional factors STAT1 and STAT3. In contrast to the decrease of Jak expression, STAT1 and STAT3 expression and activation appeared increased in lesional psoriatic skin compared with non-lesional psoriatic skin. Even more, the immunofluorescence assay of lesional psoriatic skin showed an increase in STAT1 and STAT3 phosphorylation not only in the tyrosine residue but also in the serine one throughout all the epidermal layers (Article 1, figure 2 and article 2, figure 1). It is worth noting that STAT3 is a transcriptional factor that promotes keratinocyte proliferation whereas STAT1 has an inhibitory effect. However, despite the simultaneous activation of these two transcriptional factors with opposing effects in lesional psoriatic skin, the psoriatic phenotype suggests that, in the context of a psoriatic plaque, the proliferative effect of STAT3 prevails. For this reason, this transcriptional factor was studied as a therapeutic target in the second part of this thesis.

The study of STAT3 signaling pathway in NHK showed that IL-6 and IL-20 induce its phosphorylation in the tyrosine 705 residue (Tyr705) in a Jak2 dependent manner and that this modification produces a strong transcriptional induction. The lack of commercially available specific inhibitors for the other members of the Jak family prevented us from identifying the second kinase implicated in this phosphorylation in NHK, probably Jak1 (Murray, 2007). Concurrently, other non-specific stimuli such as TPA (12-*O*-tetradecanoylphorbol-13-acetate), TNFα or UVB radiation triggered STAT3 phosphorylation in the serine 727 residue, which modulated STAT3 transcriptional activity (Article 1, figures 3 and 4). In agreement with previous studies, the global effect of this modification depended on the cellular context (Reviewed in Decker & Kovarik, 2000). These results further prove the complexity of intracellular signaling transduction, showing how different pathways can cooperate to communicate information to the nucleus through the regulation of a specific transcriptional factor (Figure 17). Therefore, STAT3 transcriptional activity in psoriatic keratinocytes is dependent on the interplay of a wide variety of mediators. Further studies will be needed in order to unveil this intricate network.

In parallel, interferon (IFN) α and el IFNγ induced STAT1 transcriptional activity through tyrosine and serine phosphorylation (Article 2).

1.2 Study of the role of adenosine in the psoriatic process

Despite the introduction of new biologic therapies, methotrexate remains one of the immunosuppressant treatments most commonly prescribed for the treatment of a variety of cutaneous conditions. Since 1951, methotrexate has been used for the treatment of moderate-to-severe psoriasis (Bangert & Costner, 2007) and it is still considered an inexpensive first-line agent backed up with many years of experience in its indications and toxicities (Warren *et al.*, 2008).

Several studies using different animal models of acute and chronic inflammation have established that adenosine mediates, at least in part, the anti-inflammatory effect of methotrexate (Cronstein *et al.*, 1993; Montesinos *et al.*, 2000; Morabito *et al.*, 1998). In addition, it has been shown that polymorphisms in enzymes involved in methotrexate-induced adenosine upregulation are likely responsible for either a good response or resistance to methotrexate (Wessels *et al.*, 2006) and caffeine, a non-selective adenosine antagonist, can decrease the effectiveness of methotrexate treatment in arthritic patients (Nesher *et al.*, 2003). However, the effects of adenosine receptor

activation on inflammatory skin diseases remain unclear. Hence, we characterized the expression pattern of adenosine receptors in psoriatic epidermis compared to normal epidermis.

In accordance with previous studies in NHK (Brown *et al.*, 2000), PCR analysis of skin biopsies confirmed that the A_{2B} receptor is the most abundant adenosine receptor in both normal and psoriatic epidermis. In addition, A_{2A} mRNA levels were also detected, although to a lesser extent, as reported in murine keratinocytes (Braun *et al.*, 2006). More interestingly, differences in the expression of these two receptor subtypes were observed between normal and psoriatic epidermis in a way that A_{2B} receptors expression was reduced in psoriatic skin, whereas A_{2A} receptors were increased (Article 3, figure 1). A_1 and A_3 were undetectable in normal and psoriatic epidermis.

Several reports have described that proinflammatory stimuli are able to modulate adenosine receptor expression (Haskó *et al.*, 2009; Morello *et al.*, 2006). Consequently, we hypothesized that the variation in adenosine receptor expression could be due to the inflammatory milieu characteristic of psoriatic plaques. We demonstrated in NHK that IFNγ, and TPA to a lesser extent, alone could reproduce the psoriatic phenotype. In addition, TNFα and IL-1β also increased A_{2A} levels whereas IFNα decreased A_{2B} expression. Other psoriatic mediators such as IL-6, la IL-17 and IL-23 had no effect (Article 3, figure 2). It is worth noting that all the tested stimuli able to modulate adenosine receptor expression did so in the same manner as they are affected in psoriatic skin, confirming, thus, that inflammatory events are able to modulate adenosine receptor expression decreasing A_{2B} and increasing A_{2A} in this cellular type.

A_{2B} is a low affinity receptor whose activation requires pathophysiological processes in which adenosine concentrations are greatly increased. Moreover, receptor density can condition its signaling capability (Haskó *et al.*, 2009). Our results suggest that in psoriatic skin A_{2B} signaling could be impaired in favor of A_{2A} receptor. In this sense, it is worth considering that A_{2A} is a high affinity receptor, therefore, its increase could have a greater impact than A_{2B} decrease. In fact, A_{2A} seems to overshadow A_{2B} anti-inflammatory effect when they coexist (Hasko *et al.*, 2008). Given A_{2A} preeminent role in adenosine anti-inflammatory effect (Ohta & Sitkovsky, 2001), it is of much interest to investigate the pathophysiological consequences of these findings.

Our preliminary results show that cAMP is the main signaling pathway associated with A_{2B} receptor activation. In this regard, our study completes the results obtained by Brown *et al.* (2000), in which

adenosine global effect was assessed. Likewise, it has been ruled out that A_{2A} receptor activation signals through cAMP in NHK (Article 3, figure 2). These results suggest that A_{2B} receptor activation may have a beneficial effect on human keratinocytes, since cAMP increase is known to have anti-inflammatory properties in psoriasis treatment (Schafer, 2012). Furthermore, A_{2A} selective and non-selective agonists were shown to inhibit IL-8 and TNFα release in TPA-stimulated NHK, suggesting a protecting role for this receptor (Article 3, figure 2).

Adenosine is a known regulator of inflammation and immunity via interaction with its specific cell-surface receptors. The preliminary results obtained in the present study demonstrate that the inflammatory conditions that are established in psoriatic plaques have an impact in adenosine receptor expression that could favor A_{2A} anti-inflammatory effects. Our group is currently working in this area in order to clarify the actions of adenosine on keratinocyte proliferation and differentiation processes as well as to elucidate the contribution of adenosine to the effect of methotrexate on skin hyperplasia.

Part 2. Search for new antipsoriatic agents

2.1 Preliminary screening

We have studied a series of molecules of natural origin in order to identify new potential antipsoriatic agents. For that purpose, we carried out a preliminary pharmacological screening in the cell line of murine macrophages RAW 264.7 in order to assess their potential anti-inflammatory effect.

Coscinolactams A and B moderately inhibited PGE_2 and NO production in stimulated macrophages. In addition, NO reduction was mediated by the inhibition of inducible NO synthase expression, enzyme that is strongly upregulated during the majority of inflammatory processes (Article 4, table 3 and figure 2).

Similarly, a series of semisynthetic derivatives of Petrosaspongiolide M, a sesterterpene isolated from the sea sponge *Petrosaspongia nigra* with a strong anti-inflammatory effect (Posadas *et al.*, 2003), were analyzed. For many years, our group has studied the anti-inflammatory properties of this family of molecules and their structure-activity relationship, having achieved very promising

results. All these derivatives are chemically characterized by the γ-hydroxybutenolide scaffold (Figure 18) and exert a potent inhibition of PGE_2 production through different mechanisms such as phospholipase A_2 inhibition or decrease of microsomal prostaglandin E synthase-1 expression (Guerrero *et al.*, 2007). Therefore, a new series of γ-hydroxybutenolide derivatives were evaluated to determine their efficacy to inhibit PGE_2 release in murine macrophages. As a result, among the 20 derivatives assayed, only 3 (14g, 16g y 18) showed a potency comparable to the leading BTH (Article 5, Table 1, figure 3). Consequently, based on these and previous results (Guerrero *et al.*, 2009; Guerrero *et al.*, 2007) we decided to continue our studies with BTH (Figure 18).

In a second stage, those compounds that produced the most interesting results in RAW 264.7 were further evaluated in NHK. Thus, the inhibitory effect of perthamides, chondroitin sulfate and BTH on TNFα and IL-8 release was confirmed. Despite the good results obtained with perthamides (Article 6, figure 6), we decided to further study the potential antipsoriatic effect of chondroitin sulfate and BTH (Figure 19). The advantages of this two compounds lay, on the one hand, in the wide clinical experience available for CS after years of use for osteoarthritis therapy and, on the other hand, in the greater potency and structural simplicity of BTH in comparison to perthamides. In addition, previous results had showed an interesting pharmacological profile of BTH *in vitro* as well as *in vivo* due to its ability to inhibit NF-κB activation (Guerrero *et al.*, 2009; Guerrero *et al.*, 2007).

2.2 Study of the antipsoriatic effect of chondroitin sulfate

CS is a glycosaminoglycan present in the extracellular matrix of a variety of tissues, including skin, where it conforms the proteoglycans. It is formed by a chain of disaccharides composed of the 1–3 linkage of D-glucuronic acid to N-acetylgalactosamine, and the disaccharide units are attached by β 1–4 galactosamine links. The galactosamine residues are sulfated in either position 4, 6 or both (Figure 20).

Being a natural component of animal tissue, CS is isolated mainly by proteolytic digestion of cartilage from bovine, porcine or marine origin. Thereby, the composition of the molecules obtained varies significantly depending on the primary source. Furthermore, oral CS is registered as a drug in many countries, whereas it is sold as a prescription-free and over the counter remedy or nutritional supplement in many others (Uebelhart, 2008). This generates a big disparity

between those preparations that have been fully registered as medicinal products, which had to fulfill severe criteria of quality and safety, and those that did not. Therefore, the diversity of all these equally named molecules may explain why the results of clinical trials differ significantly among countries (Clegg *et al.*, 2006; Hochberg, 2010; Schneider *et al.*, 2012; Wandel *et al.*, 2010). In the wake of this discussion, the origin of the active ingredients in the prescribed preparations is considered to be the most important factor ensuring quality, and thus safety and efficacy, particularly for CS, due to its extraction from different sources. In this regard, the CS used in the present study, provided by Bioibérica (Barcelona, Spain), is a highly purified mixture of chondroitin 4 and 6 sulfate of bovine origin in a concentration not less that 98%, with an average molecular weight of 15–16 kDa and a 4 sulfated/6 sulfated ratio of 2, whose anti-inflammatory capabilities have been demonstrated in several *in vitro* studies as well as in clinical trials. (Canas *et al.*, 2010; Jomphe *et al.*, 2008; Moller *et al.*, 2010; Uebelhart *et al.*, 2004).
Despite this controversy, CS has been used for many years for the treatment of osteoarthritis and is considered by the European League Against Rheumatism (EULAR) as one of the safest drugs for this condition (Jordan *et al.*, 2003). This therapeutic advantage, in addition to the preliminary clinical observation that CS could represent an effective treatment for psoriasis (Moller *et al.*, 2010; Verges *et al.*, 2005), led us to further study its effects in NHK.

Besides inhibiting TNFα and IL-8 release, CS pretreatment (200 µg/ml) decreased IL-6 and CCL27 production by NHK. The expression of all this cytokines is associated with NF-κB activation, a pivotal transcriptional factor for the pathogenesis of psoriasis (Figure 12). Accordingly, an electrophoretic mobility shift assay (EMSA) showed the ability of CS to inhibit NF-κB activation in NHK as reported in other cellular lineages (du Souich *et al.*, 2009). Thus, CS reduces the production of an array of cytokines in NHK through NF-κB inactivation, confirming its potential interest in psoriasis treatment (Article 7, figures 1-2).

Furthermore, and given the importance of STAT3 in psoriasis pathogenesis, we evaluated the effect of CS on this nuclear factor. In this sense, immunofluorescence assays as well as western blotting of the nuclear and cytosolic protein extracts showed the ability of CS to inhibit STAT3 nuclear translocation. This effect was confirmed by a luciferase assay of a STAT3 promoter that showed a significant decrease in the transcriptional activity of this nuclear factor after CS pretreatment of HaCaT cells (Article 7, figure 3). It is worth noting that this effect was independent of Tyr705 phosphorylation, indicating that CS could be acting at other levels such as the blockage

of importins or through promotion of phosphatase activity or suppressors of cytokine signalling (SOCS) activation (Figure 14).

In conclusion, our study has provided new evidence of the potential usefulness of CS for the treatment of psoriasis. The ability to inhibit two of the key signaling pathways involved in the pathogenesis of this skin disease, such as NF-κB and STAT3, in NHK (Figure 21) supports the preliminary clinical evidence regarding its potential effectiveness. Given its therapeutic profile in osteoarthritis, it could be expected that CS will provide the greatest benefits to those patients suffering from mild psoriasis, in which its outstanding tolerability will be a great advantage in comparison to other therapeutic options. The results presented here further support the need of new clinical trials in order to establish the usefulness of CS in the therapy of this skin condition.

2.3 Study of the antipsoriatic effect of BTH

As stated previously, our group has been studying for many years the anti-inflammatory properties of Petrosaspongiolide M and working on the optimization of its molecular structure through the study of semisynthetic derivatives. Thus, after the characterization of its mechanism of action, mediated by the inhibition of NF-κB (Posadas *et al.*, 2003), a series of derivatives was synthesized in order to improve its availability and pharmacological profile (Guerrero *et al.*, 2007), being part of this work included in this thesis (Article 5). Among these derivatives, BTH showed the most interesting profile, as it conserved the ability to inhibit NF-κB activation *in vitro* (Guerrero *et al.*, 2007) and showed a potent anti-inflammatory and analgesic effect in the acetic acid-induced hyperalgesia, the acute zymosan-induced inflammation in the air pouch and the collagen induced arthritis murine models (Guerrero *et al.*, 2009). Therefore, we decided to evaluate its potential antipsoriatic effect in NHK.

We first confirmed the inhibitory effect of BTH (1-10 μM) on NF-κB activation by EMSA. This effect was accompanied by a significant reduction of the levels of a series of cytokines whose release is dependent on the activation of this transcriptional factor such as IL-6 and CCL27, as well as IL-8 and TNFα (Article 8, figure 1), demonstrating, thus, the anti-inflammatory effect of BTH in NHK.

Given the recent focus on Jak/STAT3 signaling pathway in keratinocyte hyperproliferation during psoriasis pathogenesis, the effect of BTH on this pathway was evaluated. Western blotting and

immunofluorescence assays showed that BTH was able to decrease STAT3 nuclear translocation after IL-6 stimulation through the inhibition of Tyr705 phosphorylation. According to the results previously obtained in this thesis, Jak2 plays a pivotal role in this modification. Therefore, BTH could act as a Jak 2 inhibitor; however, it is not possible to conclude its selectivity with respect to other members of the Jak family as cytokine receptors can bind to one or two different Jak proteins. Finally, BTH also decreased keratinocyte proliferation after 48 hours, an effect that could be consequence of STAT3 inhibition (Article 8, figure 2).

In vitro assays are useful to dissect the mechanism of new molecules and to evaluate their potential interest. Nevertheless, *in vitro* results have to be always complemented with *in vivo* studies if these molecules are intended to reach human therapeutics. Animal models allow us to analyze the effect of such compounds when they are subject to the complex network of interactions that occurs in the whole organism in order to evaluate their efficacy and security. In our case, they also provided evidence on the suitability of BTH for topic administration, being this property an advantage for the therapy of skin diseases. Therefore, BTH antipsoriatic effect was evaluated in two animal models: the TPA-induced hyperplasia and the IMQ-induced skin inflammation models.

We used the TPA-induced hyperplasia because of its simplicity and low cost. This model allowed us to confirm the good results obtained *in vitro* with BTH, as it prevented TPA-induced lesion formation in mice. In this regard, the histological study showed a normalization of epidermal hyperplasia and leukocytic infiltrate that correlated with decreased levels of an array of cytokines and eicosanoids in skin homogenates. Thus, the levels of leukotriene B_4, PGE_2, TNFα, chemokine (C-X-C motif) ligand 1 (CXCL-1, IL-8 murine equivalent), IL-6 and IL-1β, as well as edema, were significantly lower in the BTH pre-treated mice in comparison to control mice. Finally, the inhibitory effect of BTH on NF-κB and STAT3 activation was confirmed by western blotting of the phosphorylated forms (Article 8, figures 3-4).

Additionally, and in order to confirm the potential antipsoriatic effect of BTH, we used the imiquimod-induced skin inflammation model, in which the repeated topical administration of IMQ, a Toll-like receptor (TLR) 7 and 8 agonist, for 6 consecutive days on mice skin reproduces a phenotype that shares many features with psoriatic plaques and is dependent on IL-22 and IL-23 (Van Belle *et al.*, 2012; van der Fits *et al.*, 2009). Once again, topical administration of BTH prevented the course of the disease, decreasing desquamation and erythema as well as epidermal

hyperplasia, leukocytic infiltrate and IL-23 release (Article 8, figure 5). It is worth considering, that this improvement occurred just at the site of BTH application but not at the surrounding untreated area, suggesting, thus, a local effect.

In this regard, the systemic immunologic nature of this model provided us with new evidence on the potential therapeutic advantages of BTH. The repeated application of IMQ in mice skin induces a strong stimulation of the immune system that produces a marked spleen hyperplasia, measurable at the end of the experiment (van der Fits *et al.*, 2009). As already mentioned, after topical administration, BTH prevented lesion development, but failed to improve spleen hyperplasia, further suggesting that the therapeutic effect was exerted locally. In contrast, dexamethasone, administrated as a reference treatment, decreased spleen size to a level that was even lower than that of normal mice, suggesting a strong immunosuppression. Additionally, skin atrophy could be observed beyond the dexamethasone-treated area (Article 8, figure S1).

Corticoids are the most used topic drug for the treatment of mild psoriasis in localized areas. However, adverse effects limit their use in the long term. Therefore, the search of new molecules capable of controlling lesion symptomatology with a better security profile is one of the most urging areas in psoriasis therapeutics. In the present work, we have studied the antipsoriatic effect of BTH, mediated by NF-κB and STAT3 inhibition (Figure 22). The good results obtained highlight the potential of this molecule and strengthen the interest of future studies that will determine its possible application in the therapeutics of this skin disease.

In conclusion, psoriasis is a heterogeneous pathology whose phenotype results from the interaction of a wide range of genetic and environmental factors. However, a series of common effectors mechanisms reproduce this pathology in the majority of patients. The most characteristic traits of a psoriatic lesion are the leukocytic infiltrate, attracted by the local inflammation, and the epidermal hyperplasia due to aberrant proliferation and differentiation rates. Thus, the blockade of the transcriptional factors that drive the responses involved in the pathogenesis of psoriasis entails a promising therapeutic strategy, quite unexplored still. In the present work, we have studied new molecules able to interfere with NF-κB signaling pathway, main orchestrator of the inflammatory response, and STAT3, responsible for the epidermal acanthosis and T_H17 differentiation, confirming the efficacy of this therapeutic approach in NHK cultures as well as in animal models of psoriasis. The inhibition of two of the main orchestrators of the psoriatic process

leads to a decrease of an array of mediators involved not only in the initial stages of lesion formation but also in the maintenance of the chronic inflammatory state. In this sense, the pleiotropic effect of these molecules can be an advantage respect to biologic therapies. One limitation of the currently available biologic agents is that they do not reach inside the cell to target intracellular signaling pathways. Instead, such agents selectively bind receptors or proteins on extracellular membranes or in extracellular milieu, altering the activity of targeted cells, cell-to-cell interactions, and immune signaling. Biologic agents tend to specifically target a single pro-inflammatory marker and interrupt the inflammatory cascade downstream from pro-inflammatory changes in gene transcription. To interrupt the inflammatory cascade at an earlier point, we and others have begun to explore modulation of intracellular signaling that controls inflammatory-mediator gene expression. Furthermore, the outstanding security profile of CS and the possibility of topical application of BTH make these molecules privileged candidates for the development of new therapies for the treatment of mild psoriasis. Future studies will unveil the efficacy of the therapeutic strategy postulated in the present work and the utility of BTH and CS as new antipsoriatic agents.

Overall conclusions

1. STAT1 and STAT3 expression and activation are increased in lesional psoriatic skin with respect to non-lesional psoriatic skin. Nevertheless, the expression of all Jak proteins, except Jak3, is decreased.

2. In cultured normal human keratinocytes, STAT3 Tyr705 phosphorylation induces a strong transcriptional activation whereas Ser727 phosphorylation has a modulatory effect.

3. In psoriatic epidermis there is an increased expression of A_{2A} adenosine receptors and a decreased expression of A_{2B} receptors in comparison to normal epidermis. These modifications seem to be related to the inflammatory mediators present in psoriatic plaques.

4. The study of molecules of marine origin and their semisynthetic derivatives represents a very interesting source of new potentially anti-inflammatory compounds, such as coscinolactams, perthamides and γ-hydroxybutenolide derivatives.

5. Chondroitin sulfate decreases the release of inflammatory cytokines through NF-κB inactivation in normal human keratinocytes. Furthermore, it blocks STAT3 nuclear translocation by a Tyr705 independent mechanism. These results suggest an interesting profile for the treatment of psoriasis.

6. The γ-hydroxybutenolide derivative BTH inhibits NF-κB and STAT3 signaling pathways in normal human keratinocytes cultured *in vitro* as well as in animal models of psoriasis, diminishing inflammation, leukocytic infiltrate and epidermal hyperplasia. Thereby, BTH leads to the normalization of the psoriatic phenotype and could represent a candidate for future drug research in the therapeutics of psoriasis.

Data obtained in this Thesis have shed light on the role of adenosine, Jak/STAT and NF-κB signaling pathways in psoriasis pathogenesis, as well as on the efficacy of small molecules of natural origin to ameliorate its course, suggesting the potential interest of these strategies in the development of new therapeutic approaches for this skin condition.

Figure and Table captions

1. Figure Legends

1.1 Introduction

Figure 1. Cross-section of skin and subcutaneous tissue.

Figure 2. The epidermis is organized as a stratified squamous epithelium

Figure 3. Psoriasis is a highly heterogeneous disease. Patients usually display a single form at any one time, although forms can coexist, and one form can be followed by another. Around 80% of psoriasis cases are classified as mild.

Figure 4. Plaque psoriasis is the most common form of psoriasis.

Figure 5. Several organizations, such as the Spanish Academy of Dermatology and Venereology, promote educational and consciousness raising actions about psoriasis.

Figure 6. The characteristic histological features of psoriatic skin include: thickening of the epidermis and elongation of rete ridges (acanthosis) (1), lack of stratum granulosum and retention of the nuclei in the stratum corneum (parakeratosis) (2), elongated/hyperplasic blood vessels in the papillary dermal region (3) and leukocytic infiltrate in dermis and epidermis (4).

Figure 7. Koebner's phenomenon.

Figure 8. Immunopathogenesis of psoriasis. In genetically predisposed individuals, a triggering factor activates the immunological mechanisms of the innate and adaptive immune response. Stressed keratinocytes release IL-1β, IL-6, TNFα and antimicrobial peptides (LL-37) further activating the surrounding keratinocytes and dendritic cells (DCs). In addition, the trauma can cause keratinocyte death, releasing the nuclear content into the extracellular milieu. Plasmacytoid dendritic cells (pDCs) and resident DCs are stimulated by the LL-37-DNA/RNA macrocomplexes and migrate to the lymph nodes where they present the autoantigen to T lymphocytes. There, IL-12 and IL-23 co-stimulation polarizes the immune response to T_H1 and T_H17, respectively. Mature lymphocytes return to the skin through the hypertrophic blood vessels, establishing a positive feedback loop among inflammatory DCs, lymphocytes and keratinocytes that perpetuates the

inflammatory state and hampers the physiologic keratinocyte differentiation and proliferation. NO, nitric oxide.

Figure 9. Historical evolution of the pharmacotherapy of psoriasis. A broad spectrum of therapies is available to treat psoriasis, depending on the severity of the disease. Newer biologic drugs are assessed by a 'PASI 75' score, representing the percentage of patients achieving at least a 75% reduction in their Psoriasis Area and Severity Index (PASI).

Figure 10. NF-κB Signaling pathway. In its latent state, p65-p50 dimers remain in the cytosol forming a complex with the inhibitor IκB. Binding of a wide variety of extracellular mediators activates the IKK kinase that phosphorylates IκB. This modification triggers the dissociation of the IκB-NF-κB complex and the subsequent IκB degradation by the proteasome 26S. The free p65-p50 dimers translocate to the nucleus were they bind specific DNA sequences, initiating the transcription of an array of genes among which is the IκB. The newly synthesized inhibitor is able to dissociate NF-κB-DNA complexes and bring the p65-p50 back to the cytosolic latent state. Thereby, the system is susceptible to be stimulated again if the stimulus persists.

Figure 11. NF-κB is a central regulator of the transcriptional responses to a wide variety of physiological and environmental stimuli. Signals ranging from pro-inflammatory cytokines to stresses including reactive oxygen species (ROS), ultraviolet light (UV), DNA double-strand breaks and engagement of antigen receptors lead to activation of NF-κB that initiates the transcription of a wide variety of genes. These transcriptional programs have far-reaching biological consequences and include survival factors, growth factors, cytokines, chemokines and numerous core mediators of adaptive and innate immunity.

Figure 12. NF-κB plays a pivotal role in the pathogenesis of psoriasis. The majority of the cytokines produced during the psoriatic process depend on NF-κB activation. Furthermore, NF-κB is essential for the activation and differentiation of immune cells as well as for their maintenance in the inflammatory focus.

Figure 13. Jak/STAT signaling pathway. Binding of the specific ligand triggers receptor dimerization (1), allowing the mutual phosphorylation and activation of the associated Jak (2). As a consequence, the receptor is phosphorylated by Jak (3) creating a docking site for two subunits of STAT that are subsequently phosphorylated (4). This induces STAT dimerization and separation

from the receptor (5). The recently released STAT dimers translocate to the nucleus were they promote the transcription of specific genes.

Figure 14. Jak/STAT signaling pathway is regulated by a variety of inhibitory proteins. The JAK/STAT pathway can be affected by several negative feedback loops, including suppressors of cytokine signaling (SOCS) proteins, protein inhibitor of activated STAT (PIAS), and phosphatases. SOCS proteins can affect JAK kinase activity, STAT binding to the receptor, and protein stability. PIAS inhibits STAT transcription.

Figure 15. STAT3 integrates the signals of various stimuli and coordinates cellular responses that include a wide variety of physiological processes.

Figure 16. Molecular structure of adenosine.

1.2 Results and discussion

Figure 17. STAT3 total transcriptional activity depends on the interplay of several signaling pathways.

Figure 18. Molecular structures of Petrosaspongiolide M and BTH. The γ-hidroxybutenolide scaffold characteristic of this family of compounds is highlighted in blue.

Figure 19. During the first stage of our study, the candidate molecules were subjected to a screening process based on their potential anti-inflammatory effect, firstly in cell lines and thereafter in NHK. BTH and CS were selected to further study their antipsoriatic activity.

Figure 20. Chemical structure of a chondroitin sulfate chain unit. The positions that can be sulfated are highlighted in blue. Chondroitin-4-sulfate: R_1 = H; R_2 = SO_3H. Chondroitin-6-sulfate: R_1 = SO_3H; R_2 = H.

Figure 21. The ability to inhibit NF-κB and STAT3 in NHK suggests that CS could represent a new therapeutic alternative for the treatment of psoriasis.

Figure 22. BTH pretreatment inhibits NF-κB and STAT3 signaling pathways, diminishing the inflammation, leukocytic infiltrate and epidermal hyperplasia leading to the normalization of the psoriatic phenotype.

2. Table Legends

2.1 Introduction

Table 1. Results from the most recent meta-analysis of the psoriasis susceptibility loci. A major involvement of NF-κB (shown in blue) and STAT3 (shown in pink) signaling pathways can be observed. Some genes participate in both pathways (shown in purple). **Odds ratio:** ratio of the odds of an event occurring in one group to the odds of it occurring in another group. DC, dendritic cells; MHC-1, major histocompatibility complex 1; SNP, single-nucleotide polymorphism. Bibliography: Bijlmakers *et al.* (2011); Capon *et al.* (2008); Capon *et al.* (2012); Ellinghaus *et al.* (2012); Li *et al.* (2006); Perera *et al.* (2012); Strange *et al.* (2010); Stuart *et al.* (2010); Tsoi *et al.* (2012).

Table 2. Binding affinities of the agonists and antagonists used in the present thesis for the four adenosine receptor subtypes in comparison to adenosine. Ki, affinity constant obtained by radioligand binding studies. Data provided by Tocris Bioscience (R & D Systems).

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Anexo

VNIVERSITAT DE VALÈNCIA
Vicerectorat d'Investigació i Política Científica

D. Fernando A. Verdú Pascual, Profesor Titular de Medicina Legal y Forense, y Secretario del Comité Ético de Investigación en Humanos de la Comisión de Ética en Investigación Experimental de la Universitat de València,

CERTIFICA:

Que el Comité Ético de Investigación en Humanos, en la reunión celebrada en el día de hoy, una vez estudiado el proyecto de investigación titulado:

"Mecanismos implicados en la protección y regeneración tisular en afecciones cutáneas con componente inflamatorio",

cuya investigadora principal es Dña. Mª Carmen Montesinos Mezquina,

ha acordado informar favorablemente el mismo dado que se respetan los principios fundamentales establecidos en la Declaración de Helsinki, en el Convenio del Consejo de Europa relativo a los derechos humanos y cumple los requisitos establecidos en la legislación española en el ámbito de la investigación biomédica, la protección de datos de carácter personal y la bioética.

Y para que conste, se firma el presente certificado en Valencia, a dieciocho de febrero de dos mil nueve.

Fernando Verdú Pascual
Profesor Titular de Medicina Legal y Forense

VNIVERSITAT DE VALÈNCIA
Facultat de Medicina i Odontología
Valencia España

Certifico la precisión e integridad de este documento
2009.02.18 17:06:22 +01'00'

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DR. PEDRO CARRASCO SORLÍ
Presidente del Comité Ético de Experimentación y Bienestar Animal de la Universitat de València

CERTIFICA:

Que, reunido el Comité Ético de Experimentación y Bienestar Animal el día 9 de febrero de 2009, acordó informar FAVORABLEMENTE la realización en sus instalaciones del protocolo de experimentación animal, presentado en el proyecto titulado **"Mecanismos implicados en la protección y regeneración tisular en afecciones cutáneas con componente inflamatorio"**, presentado por la Dra. Mª Carmen Montesinos Mezquita.

En Valencia, a 4 de marzo de 2009.